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Novel Nitroxide Resuscitation Strategies in Experimental Traumatic Brain Injury

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14. ABSTRACT

In this funding period, we developed a new model of combined experimental traumatic brain injury (TBI) plus hemorrhagic shock to study the effect of resuscitation with novel polynitroxylated colloids or hemoglobins in a series of 18 experimental studies carried out over 4 years. The most important finding was that a novel polynitroxylated, pegylated bovine cell-free hemoglobin (PNPH, a 4% solution in saline) served as a small volume resuscitation fluid that improved brain tissue oxygen levels and was neuroprotective. This neuroprotection was not seen with conventional resuscitation solutions such as lactated Ringers, Hextend, or hypertonic saline. A polynitroxylated albumin solution was also not as effective as PNPH. In addition, using a series of in vitro models of primary rat hippocampal neuronal culture, including glutamate/glycine toxicity and neuronal stretch injury, PNPH was shown to be uniquely neuroprotective in vitro—while native bovine hemoglobin was (as expected) neurotoxic. We believe that these findings are important and suggest that covalent modification of hemoglobin with the combination of antioxidant nitroxides and polyethylene glycol produces a potential paradigm shift in the development of new hemoglobins as resuscitation fluids. PNPH deserves additional study to move it to IND for TBI resuscitation.

15. SUBJECT TERMS
Traumatic Brain Injury, hemorrhagic shock, resuscitation, neuroprotection, nitroxide, hemoglobin, blood substitute

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INTRODUCTION:

The work funded by this grant has been focused on the development and testing of novel resuscitation approaches to the combination of traumatic brain injury (TBI) and hemorrhagic shock (HS) (TBI+HS) using an experimental model in mice. The problem of TBI + HS has taken on great importance in the wars in Iraq and Afghanistan, where blast-induced TBI has become a leading cause of morbidity and mortality for our soldiers. The novel nitroxide-based resuscitation fluids—polynitroxylated albumin (PNA) and polynitroxylated pegylated hemoglobin (PNPH) combine—in a single molecule—the potential contributions of efficient volume expansion by a colloid- or hemoglobin (Hb)-based solution along with the potent antioxidant effects of multiple nitroxide moieties. The program project had four key components, #s 1-3 at the University of Pittsburgh School of Medicine/Carnegie Mellon University, Pittsburgh, PA and #4 at Synzyme Technologies, Irvine, CA. The research sites include 1) the Safar Center for Resuscitation Research, University of Pittsburgh (site of the experimental TBI+HS model, Patrick M. Kochanek, MD, PI), 2) the Pittsburgh NMR Center for Biomedical Research, Carnegie Mellon University (site of *in vivo* testing of the effects of TBI+HS and resuscitation on cerebral blood flow (CBF) using magnetic resonance imaging (MRI), Chien Ho, PhD, PI), 3) the Pittsburgh Center for Free Radical and Antioxidant Health, University of Pittsburgh (using a battery of markers of oxidative stress applied in brain injury), Valerian Kagan, PhD, PI and Hülya Bayır, MD, Co-I), and 4) SynZyme Technologies, Irvine, CA (developer of the nitroxide-based solutions, Carleton Hsia, PhD and Li Ma, PhD, PIs).

In year 1, a reproducible model of TBI+HS in mice was established. Using this model, HS (90 min) after TBI markedly increased hippocampal neuronal death.

In years 1-2, we also established oxidative lipidomics methods. In year 2 we studied CBF and showed that it was critically reduced during TBI+HS. In year 2, we also tested several small volume resuscitation fluids including Hextend (HEX), hypertonic saline (HTS), and PNA. Both PNA and HEX required smaller resuscitation volumes to achieve the desired target mean arterial blood pressure (MAP) than Lactated Ringers (LR) or HTS, and although this finding could be valuable in reducing the amount of field resuscitation fluid required for a medic, neither agent reduced neuronal death.

In year 3 we explored the novel Hb PNPH in our TBI + HS model and observed exciting neuroprotective effects, in a number of preliminary and definitive studies.

In year 4, we completed an extensive battery of work focused on PNPH—with both *in vivo* studies of survival, hemodynamics, neuropathological outcomes, and brain tissue oxygen levels ($PbtO_2$), and *in vitro* studies, all showing remarkable neuroprotective effects of this novel agent.

We published three manuscripts (1-3), have a fourth in revision (4), presented 20 abstracts related to this work at meetings including ATACCC, the Society of Critical Care Medicine, the 12th Symposium on Blood Substitutes, the International Society of Magnetic Resonance and Medicine, and the National and International Neurotrauma Societies Congress, among others (11-30). We are completing work for 5 additional manuscripts in preparation related to this project (6-10). A complete list of publications and abstracts is provided.

Based on this work we submitted to the CDMRP a grant application to support IND development for PNPH for TBI resuscitation which received a remarkable scientific score of 1.0. It was not funded. However, in this most recent cycle, we (P. Kochanek, MD, PI) have submitted a revision of that application again to the CDMRP, carefully addressing the critique. Given the highly promising nature of the findings, we also partnered with SynZyme (C. Hsia, PhD, PI) to submit to the NIH, an SBIR (U44) grant to support acquisition of an IND for PNPH for TBI resuscitation. We are hopeful that these will be successful since we believe that PNPH is a strong candidate for clinical development for TBI

resuscitation, and we believe that it is important to be able to develop PNPH through to IND for combat casualty resuscitation of severe TBI victims. This progress report describes all of the work accomplished during the entire 4 year funding period (Feb 13, 2008-Feb 12, 2010).

BODY:

A complete listing of the studies carried out in this project is provided in Table 1. For the convenience of the reader, we have categorized studies as "M" mechanistic or "T" treatment.

Table 1. Matrix of work carried out in years 1-4

Study	Year	Study composition	Outcomes
M1	1-2	Model development—TBI + volume controlled HS (MAP 35-40 mmHg)	Fluid requirements; physiology; 7 d neuropathology
M2	2	MRI assessment	CBF
T1	2-3	PNA vs HEX, LR, or HTS	Fluid requirements; physiology; 7 d neuropathology
T2	2-3	PNA vs HEX, LR, albumin or PNA+Tempol	Markers of oxidative stress
T3	2-3	PNA vs HEX, LR, albumin, or PNA+Tempol	Fluid requirements; physiology; 7 d neuropathology
T4	3	PNPH vs HEX or LR	Fluid requirements; physiology; 7 d neuropathology
T5	3	Top load; PNPH vs stroma free Hb	MAP
M3	3	Naïve, TBI, and TBI+HS	$PbtO_2$
M4	3	More severe insult—TBI + pressure controlled HS (MAP 25-27 mmHg)	Neuropathology and $PbtO_2$
T6	3	PNPH vs LR	$PbtO_2$ and 24 h neuropathology
M5	3	MRI; naïve, TBI alone, TBI+HS	Brain edema
T7	3-4	TBI alone PNPH vs LR	Physiology; 7 d neuropathology
M6	3	Naïve, TBI, and TBI+HS	ICP
T8	3-4	*In vitro* TBI; PNPH, PegHb, stroma free Hb	Neuronal death/neurotoxicity from Hb exposure (LDH, MTT)
T9	4	*In vitro* TBI; PNPH, PegHb, stroma free Hb	Glutamate/Glycine excitotoxicity (LDH, MTT)
T10	4	*In vitro* TBI; PNPH, PegHb, stroma free Hb	Neuronal stretch (LDH, MTT, propidium iodide labeling, MAP-2 immunofluorescence
M7	1-4	Mechanistic studies on oxidative stress and effects of nitroxides and PNPH	Oxidative lipidomics, *in vitro* studies of oxidative stress related to PNPH
M8	4	Functional outcome, long-term 21d neuropathology after TBI+HS in mice	Morris water maze, Lesion volume, hemispheric tissue loss assessments

M=Model; T=Treatment; TBI = traumatic brain injury; HS=hemorrhagic shock; MAP=mean arterial pressure; PNA=polynitroxylated albumin; HEX=Hextend; PNPH=polynitroxylated pegylated hemoglobin; Hb=hemoglobin; CBF=cerebral blood flow; $PbtO_2$=brain tissue oxygen concentration; ICP=intracranial pressure; LDH=Lactate dehydrogenase; MTT =(3-[4, 5-dimethylthiazol-2-yl-]-2, 5-diphenyltetrazolium bromide); MAP-2=Microtubule-associated protein-2

<u>Study M1</u>. Modeling combined TBI + HS in mice

Much of the work in years 1-2 was devoted to developing a reproducible mouse model of combined TBI + HS. That work was presented in abstract form at several major scientific meetings (11-15) and the model was published as a full manuscript in the *Journal of Neurotrauma* in 2009 (1). Of note, Critical Care Medicine fellow Dr. Alia Dennis received the *In training award* from the Society of Critical Care Medicine in 2007 for her work on that paper recognizing her as the top trainee at the congress. We then used this mouse model of TBI + HS in years 2 through 4 for studies of both PNA and PNPH. We also developed a more severe pressure controlled HS model of combined TBI+HS—discussed later. One of the surprising but important finding of this work was that 90 min of HS at the level of mean arterial pressure (MAP) 35-40 mmHg was necessary to produce an exacerbation of neuronal death in hippocampus after TBI. A period of 60 min of HS was insufficient. The general paradigm, thus, for the *in vivo* studies that we performed that were funded by this grant are provided in **Figure 1**—where a HS phase is followed by a "Pre-hospital" phase, where the test solution is administered, followed by a "Hospital" or "Definitive Care" phase, where the shed blood is returned and 100% oxygen is administered, mimicking care in the emergency department or corps area support hospital (CASH).

<u>Study M2</u>. Serial non-invasive assessment of CBF after combined TBI + HS in mice

We also studied the combined effect of HS on cerebral blood flow (CBF) after experimental TBI using this model as assessed by serial magnetic resonance imaging (MRI) using arterial spin labeling. In collaboration with scientists at the Pittsburgh NMR Center for Biomedical Research at Carnegie Mellon University we applied the state-of-the-art non-invasive arterial spin labeling (ASL) to assess regional CBF serially in each phase of the model. ASL uses endogenous arterial water as a tracer by labeling the water proton spins via a low power radio frequency field. Control and labeled images are subtracted and a CBF map is calculated based on known constants. **We carried out a comprehensive study showing its utility in combined TBI + HS in our model and demonstrated that HS indeed produces critical CBF levels after TBI –particularly in cortex and hippocampus. This allows the study of CBF after combined TBI plus HS, as proposed in this application**. CBF was quantified in cortical, hippocampal, thalamic, and hemispheric regions of interest. The advantages of this method are that 1) it is non-invasive, 2) can be performed serially, and 3) provides a map of CBF rather than assessing a single region. Our data (**Figure 2**) show that **HS at an MAP of ~35-40 mmHg for 90 min exacerbates the CBF reduction after CCI, consistent with the histopathology.** HS alone for 60 min at this BP level produced only a modest CBF reduction—consistent with the lack of neuronal death seen for HS alone at this shorter interval. Our findings were presented at three scientific meetings (12, 14, 15) and a full manuscript of this work is in preparation (6).

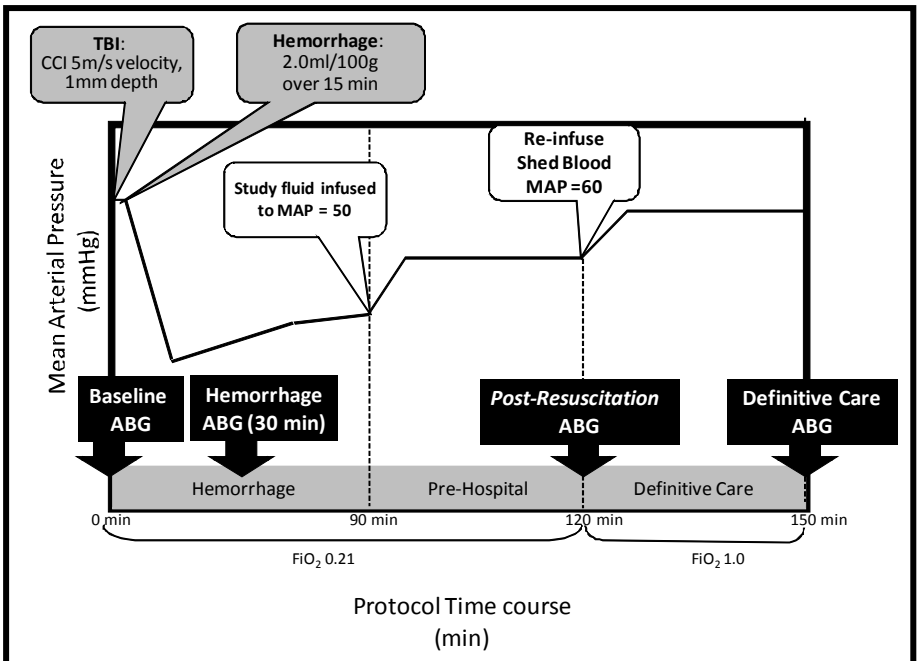

Figure 1. Diagram depicting the time course of the experimental protocol used for studies T1-T. CCI, controlled cortical impact; TBI, traumatic brain injury; MAP, mean arterial blood pressure; ABG = Arterial blood gas.

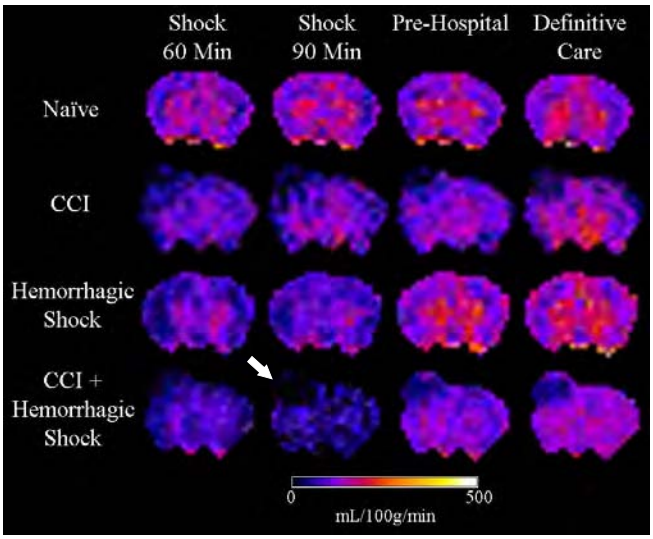

Figure 2. Effect of HS on CBF after TBI in mice assessed via MRI. CBF is stable over time in naïves. After TBI (CCI) a CBF reduction is seen only in the contused left cortex. HS alone produces a mild global CBF reduction, while TBI+HS produces a marked CBF reduction (arrow) after HS. Contusional swelling is seen after resuscitation with HEX—the military standard of care for TBI+HS.

<u>Studies T1-T3</u>. Novel colloid-based resuscitation strategies using PNA: We showed that resuscitation in our mouse model of TBI+HS using PNA or HEX significantly reduced fluid requirement vs either LR or HTS. The T1 study compared LR, HEX, HTS, and PNA in our model, while the T2 and T3 study compared HEX, LR, 10% albumin, PNA, and PNA+ the antioxidant tempol, assessing oxidative stress markers (T2) and neuropathology (T3), respectively. In each case the colloids outperformed the crystalloids with regard to fluid volume required after TBI+HS. These findings bring into question the conclusions of the recent SAFE study comparing albumin and normal saline in TBI resuscitation (Myburgh et al, *N Engl J Med*, 2007). The SAFE study did not evaluate field use of

colloids, rather only ICU use after resuscitation. Also, it did not focus on patients with TBI+HS, and did not target patients either in shock or with low serum albumin values. **Our work suggests that there is merit to colloids in resuscitation of victims of TBI+HS and is supported by recent work both in the lab (Baker et al, *J Trauma*, 2008) and ICU (Bernard et al, *J Trauma*, 2008). This is important in that the current standard of care for combat casualty care field resuscitation of TBI+HS is the colloid HEX (Holcomb, *J Trauma*, 2003).** However, although we found the colloids PNA and HEX to require smaller volumes than LR or hypertonic saline to achieve target MAP during a simulated "Pre-hospital" resuscitation phase, we did not observe added neuroprotection by the antioxidant colloid PNA in our model of TBI+HS. That work was presented at several scientific meetings by Critical Care Medicine Fellow Dr. Jennifer Exo (16, 19, 23) and was also published in 2009 as a manuscript in the *Journal of Neurotrauma* (2). **Data from this work in year two were presented in piror reports and are not presented in lieu of the full publication of this work (2).** The inability of either colloid to enhance neuroprotection prompted us to test the novel Hb PNPH in our model.

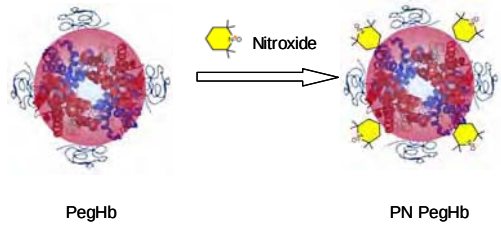

Figure 3. Schematic of PNPH. Poly nitroxide (PN, yellow) and polyethylene glycol (Peg, blue) moieties decorate the Hb surface. The nitroxides mitigate reactive oxygen species, attenuating nitric oxide (NO) consumption, and oxidative injury to the microcirculation and the Hb molecule itself. PNPH has 12-14 nitroxides per Hb. Pegylation yields super colloid effects increasing endothelial NO production and enhancing volume expansion with less brain edema.

PNPH: In addition to the colloid-based approach with PNA that we studied, we also explored a Hb-based nitroxide. Several blood substitutes have shown potential in experimental TBI+HS. TBI+HS may represent a special opportunity for resuscitation with Hb solutions in that benefit on intracranial pressure (ICP), $PbtO_2$, or neuropathology can be achieved with an optimized blood substitute (Patel et al, *J Trauma*, 2006; Rosenthal *J Neurosurg*, 2008). **However, clinical testing of blood substitutes has failed to show benefit; these trials have actually shown increases in mortality (Natanson et al, *JAMA*, 2008). A better Hb-based resuscitation solution would represent a significant advance. The data presented in this report show remarkable neuroprotective properties of PNPH both in vitro and in vivo and support our desire to move PNPH to an IND for TBI resuscitation.** There are problems with first generation Hbs, i.e., 1) binding and consumption of nitric oxide (NO) resulting in arterial hypertension and a compromised microcirculation, 2) inability to optimize tissue oxygen delivery, and 3) auto-oxidation and pro-oxidant potential, with exacerbation of oxidative stress. **Nevertheless, in TBI+HS, the huge tactical advantages of being able to use an optimized, single dose, small volume resuscitation solution in austere environments or during combat cannot be underestimated.** There is a vital need to develop second or third generation blood substitutes (Alayash, *Nat Rev Drug Discov*, 2004)—and, based on the need for a small volume, neuroprotective, and antioxidant resuscitation solution that also restores oxygen delivery, minimizes brain edema, improves CBF, and exhibits favorable effects in the microcirculation, **blast-induced TBI+HS defines a key target for such an agent in combat casualty care.** As shown in **Figure 3,** PNPH with its nitroxide and Peg moieties has advantages over conventional Hb solutions. Each component of PNPH confers potential benefit. Nitroxides are synthetic, highly stable free radicals that react with biological free radicals *in vivo* and protect cells from oxidative insults. The nitroxide free radical (Tempol) reduced brain damage in rats subjected to weight drop TBI (Beit-Yannai et al, *Brain Res*, 1996). The PN-colloid, PNA in combination with the free nitroxide Tempol, improved survival in a model of HS in rats (Kentner et al, *J Trauma*, 2002), and PNA was a small volume resuscitation solution in our TBI+HS model as described above (2). Pegylated Hb (Peg-Hb) is an excellent platform upon which to build an optimized resuscitation solution. Vasoactive effects of free Hb may be caused by NO depletion (Resta et al, *J Appl Physiol*, 2002), excess oxygen delivery at the Hb-endothelial interface, or both (Tsai et al, *Am J Physiol*, 2003). Pegylation reduces P50 of the Hb from ~28 mmHg in whole blood to between <8 and 15 mmHg (Winslow, *Respir Physiol Neurobiol*, 2007). This could compensate for potential excess tissue oxygen delivery from free Hb—with microcirculatory dysfunction (Winslow, *Biochem Biophys Acta*, 2008). Pegylation confers other favorable properties including a colloid

effect—enhancing fluid flux out of the brain interstitial space, reducing Hb degradation, and prolonging half-life. **Details on the properties and production of PNPH are provided in the Progress Report from SynZyme Technologies.**

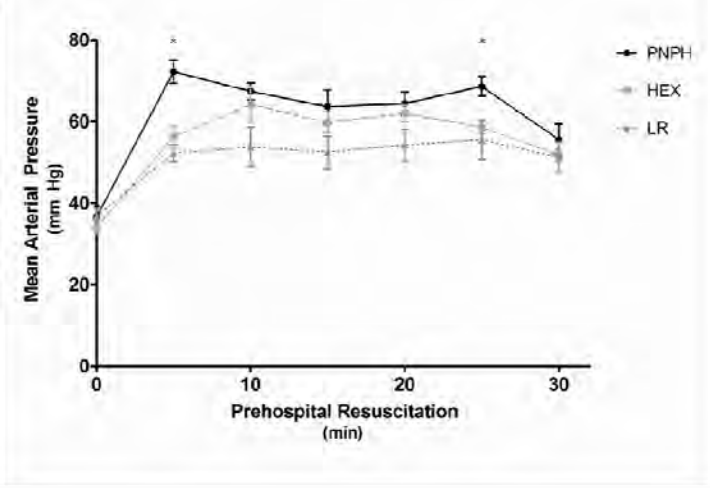

Figure 4. Time course of the response in MAP to resuscitation fluid during the 30 min *Pre-hospital* resuscitation phase for each group. Data indicate mean +/- SEM at each time point, n=5 per group. (* p<0.05, ANOVA)

Study T4. Preliminary assessment of PNPH as a novel pre-hospital resuscitation fluid in experimental TBI+HS: In this initial study in our TBI+HS mouse model, we evaluated the effect of PNPH (n=6), LR (n=8) and HEX (n=5) in pre-hospital resuscitation. Isoflurane anesthetized C57BL6 mice were subjected to CCI (5 m/s, 1 mm depth) followed by HS (2cc/100g, [~30% blood volume], MAP 35-40 mmHg) for 90 min. MAP was then maintained >50 mmHg for 30 min with PNPH, LR, or HEX. After 30 min, shed blood was infused. We apportioned mice to each treatment in order to generate 5 survivors to 7 d. MAP was monitored. Resuscitation volumes were recorded. Mice were allowed to recover and 7 d neuropathology was examined using hematoxylin and eosin (H&E) and Fluoro-Jade C (FJC) staining of coronal brain sections taken through the lesion at the level of the dorsal hippocampus. Mortality did not differ significantly between groups. **Resuscitation with PNPH (0.18 ± 0.05 ml) required less volume than LR (0.96 ± 0.28 ml)** (p < 0.05). **This represented a 5-fold reduction in the resuscitation volume required to achieve target MAP (50 mmHg) in the pre-hospital phase comparing PNPH vs LR.** PNPH (64.4 ± 2.9 mmHg) but not HEX (58.8 ± 2.9) also produced higher pre-hospital mean MAP vs LR (50.4 ± 2.9, p<0.05). Similarly, PNPH but not HEX exhibited a higher pre-hospital peak MAP vs LR (p< 0.05) (**Figure 4**). **PNPH-resuscitated mice also had fewer FJC+ degenerating neurons in the CA1 region of the hippocampus vs HEX or LR (p<0.05), suggesting important neuroprotective effects of PNPH as a pre-hospital resuscitation solution in TBI+HS (Figure 5). This neuroprotective effect of PNPH seen on FJC-staining in this initial study was also suggested in H&E-stained brain sections, although our sample size**

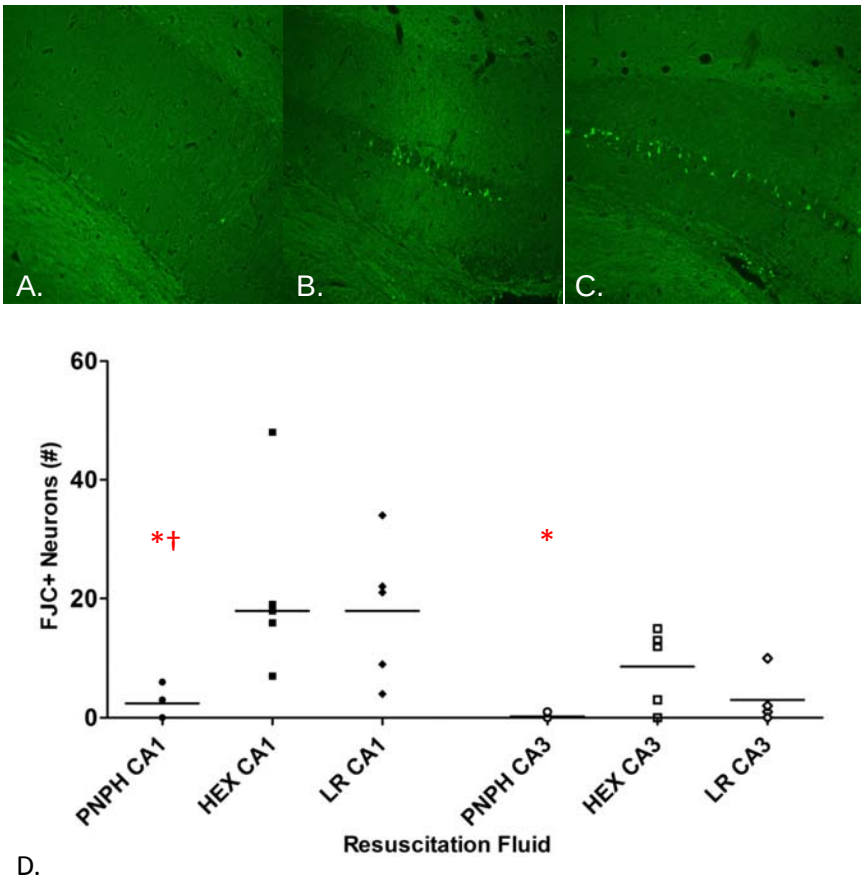

Figure 5. Photomicrographs of representative sections of CA1 stained with Fluoro-Jade C depict degenerating neurons in mice resuscitated with (A) PNPH, (B) HEX, or (C) LR. Plot (D) of number of FJC positive neurons in CA1 and CA3 for each group with median shown. (p<0.05, PNPH vs. *HEX, and †LR)

was limited and a trend toward reduced damage was seen in the PNPH group vs LR or HEX. The neuroprotective effect of PNPH in our model may have resulted from enhanced oxygen delivery to the injured brain, improved MAP during the pre-hospital phase, a reduction in brain edema related to the small resuscitation volume required with its use, or other effects. In addition, PNPH is prepared in the carboxy-(CO) Hb form and some studies have suggested beneficial effects of low doses of carbon monoxide against apoptosis (Jin and Choi, Proc Am Thorac Soc, 2005). To place our findings in context, we have evaluated HTS, LR, and HEX in this model in the T1 study and found that none of these fluids showed benefit on CA1 neuronal survival in hippocampus after TBI+HS. **This suggested special neuroprotective benefit of PNPH. This work was presented at several scientific meetings (21, 22) and also in a plenary presentation at the 12th Symposium on Blood Substitutes (24). It is in revision as a manuscript in the journal *Critical Care Medicine* (4) and was carried out by Critical Care Medicine fellow Dr. David Shellington who is currently an officer in the United States Navy.**

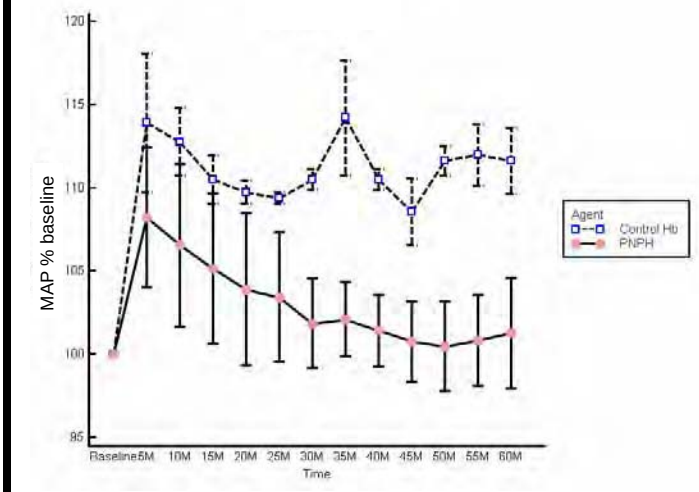

Figure 6. Top-load study: MAP (% baseline) over time after infusion of 10% blood volume study solution (PNPH or stroma-free human Hb) to healthy C57/BL6 male mice (p = 0.07, n=3/group).

Study T5*, *Effect of PNPH on MAP in naïve mice—classical top-loading studies: A concern with stroma free Hb relates to what has been termed its "vasoactivity." This effect likely results from NO consumption by Hb and possibly local hyperoxia with compensatory vasoconstriction. Although there is controversy over the etiology, it is believed that this property is deleterious and contributed to the failure of clinical translation of hemoglobin blood oxygen carriers (HBOCs). We carried out pilot studies with PNPH, examining its effect on MAP in a conventional 10% top load study in isoflurane anesthetized naïve mice. Hypertension was sustained after top-loading with stroma free human control Hb. In contrast, hypertension was only transient after PNPH suggesting less NO consumption and a safer microcirculatory profile for resuscitation (**Figure 6**).

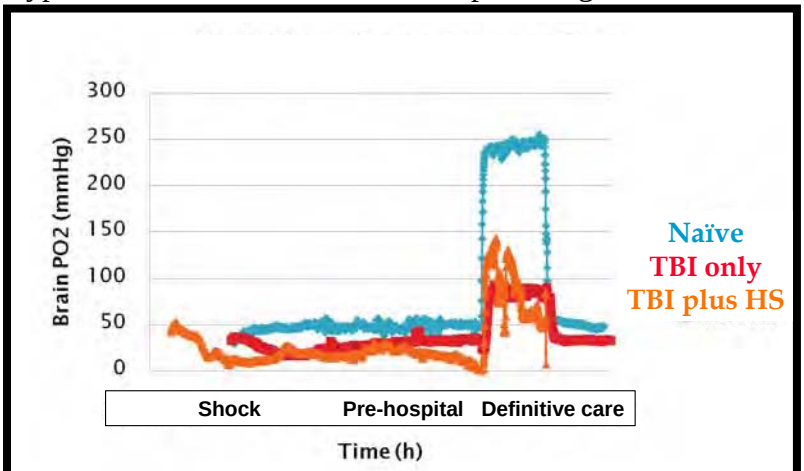

Figure 7. Hippocampal $PbtO_2$ vs time in mice. The naïve (blue) was anesthetized but not exposed to either TBI or HS. Normal $PbtO_2$ values on room air of ~50 mmHg were seen. During the "definitive care" phase, FiO_2 was increased to 1.0 producing a robust response. CCI alone (red) produced a moderate $PbtO_2$ reduction to ~30 mmHg. CCI+HS (orange) reduced $PbtO_2$ below the critical threshold (~10 mmHg). The response to raising FiO_2 to 1.0 was blunted after TBI.

Study M3*, *Continuous monitoring of hippocampal $PbtO_2$ in mice: To compliment the assessment of CBF during HS and resuscitation in our model, it will be important to monitor the effect of resuscitation with various therapies on $PbtO_2$. Normalization of both CBF and $PbtO_2$ during pre-hospital resuscitation is a goal. We carried out pilot studies in our model implanting a microelectrode (Unisense, 50 μM) through the cortex into the underlying hippocampus (bregma -2.5 AP, -2.0 ML, depth 2.0 mm) in naïve mice, and in mice after either CCI or CCI plus HS. This approach is feasible in mice (**Figure 7**). Anticipated $PbtO_2$ levels for normal (naïve) mice are seen (~50 mmHg). We also noted $PbtO_2$ levels of ~25-30 mmHg on the side ipsilateral to the contusion after CCI alone. That level of

$PbtO_2$, which is less than naïve—but above the critical threshold of ~10 mmHg (24), agrees with the observed histopathology at this level of injury severity—i.e., CCI alone does not produce neuronal death in hippocampus.

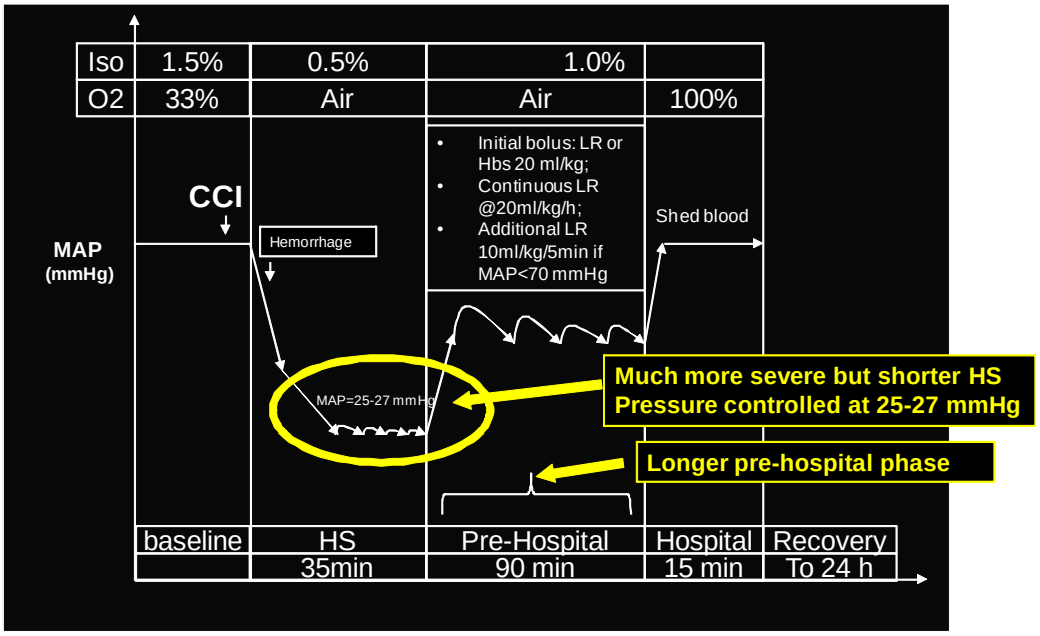

Figure 8. Protocol for pressure controlled model of TBI+HS in mice. This model is more severe than the volume control model shown in Figure 1 with an MAP of 25-27 mmHg and produces critical $PbtO_2$ levels in hippocampus during the HS phase. This model provided a perfect target to test PNPH vs LR (as shown in study T6).

In contrast, $PbtO_2$ is further reduced after TBI by HS in our model—to a level below the 10 mmHg critical threshold (Figaji et al, *Neurosurgery*, 2008). This is consistent with the fact that combined injury results in CA1 neuronal death. We have used $PbtO_2$ in >50 mice, and it has performed consistently with the data shown. We then built upon these pilot studies to compare the effects of resuscitation with LR vs PNPH in our model, i.e., **we evaluated the effect of PNPH vs LR on recovery of $PbtO_2$ in our mouse model of TBI+HS as described in study T6 below.**

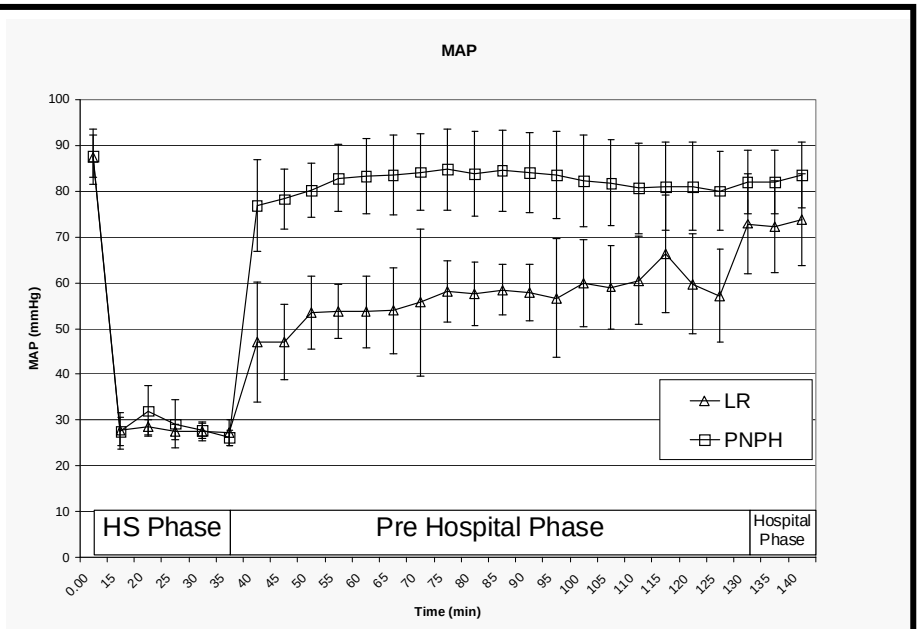

Figure 9. MAP during HS and resuscitation in mice treated with LR or PNPH. MAP was nearly normalized in PNPH group at all time points during the pre-hospital phase ($p < 0.05$ PNPH vs LR).

<u>Studies M4 and T6.</u> Effect of PNPH vs LR on recovery of hippocampal $PbtO_2$ after severe pressure **controlled TBI+HS in mice:** With brain tissue monitoring in place, we carried out another study assessing the effect of PNPH vs LR on systemic physiology, hippocampal $PbtO_2$ and 24 h neuropathology after TBI+HS. **For this work, we increased the severity of our model to a very severe MAP target during HS—namely severe pressure controlled HS clamping MAP at 25-27 mmHg for 35 min.** In pilot studies we showed that this results in a more severe acute decline in $PbtO_2$ and greater neuropathological damage. As discussed later, we have followed up to also fully characterize this more severe pressure controlled model of TBI+HS in mice as described in study M7. To better define the effect of PNPH vs LR on both hippocampal $PbtO_2$ during resuscitation, and neuropathological damage after recovery in this more severe model, we used a 90 min pre-hospital resuscitation

phase as outlined in the protocol in **Figure 8. Resuscitation with PNPH produced significantly higher MAP than LR (Figure 9)—with near normalization during the entire pre-hospital phase despite a dramatically lower resuscitation volume requirement with PNPH (50±4 mL/kg) vs LR (205±20 mL/kg). Also, systemic variables such as arterial pH, base deficit and lactate improved better in the PNPH vs LR groups (data available upon request). This is a favorable hemodynamic resuscitation profile for PNPH that argues strongly against deleterious extracerebral consumption of NO by PNPH.** In this study we also continuously monitored hippocampal $PbtO_2$ during HS and resuscitation, and PNPH again appeared to confer favorable effects, improving $PbtO_2$ during the final ~30 min of the pre-hospital resuscitation phase ($p<0.05$ vs LR, **Figure 10**). Finally, PNPH also attenuated hippocampal neuronal death in CA1 assessed at 24 h after the insult, replicating the neuropathological benefit shown in the more mild HS insults carried out in study 4 (**Figure 11**). **Thus, improved oxygen delivery is one mechanism by which PNPH may be neuroprotective after combined TBI+HS. Other mechanisms that should be pursued to more fully understanding the mechanistic underpinnings of the neuroprotection of PNPH include antioxidant effects, vascular (blood-brain barrier) damage, CBF, reduced brain edema, sparing of NO with improved microcirculatory flow and reduced systemic derangements, and possibly effects of the CO moiety.** This study was carried out by Safar Center fellow Dr. Xianren Wu who presented the work at the meetings of the American Society for Anesthesiology and the National Neurotrauma in 2009 (26, 29) and it is currently being written up for publication (8).

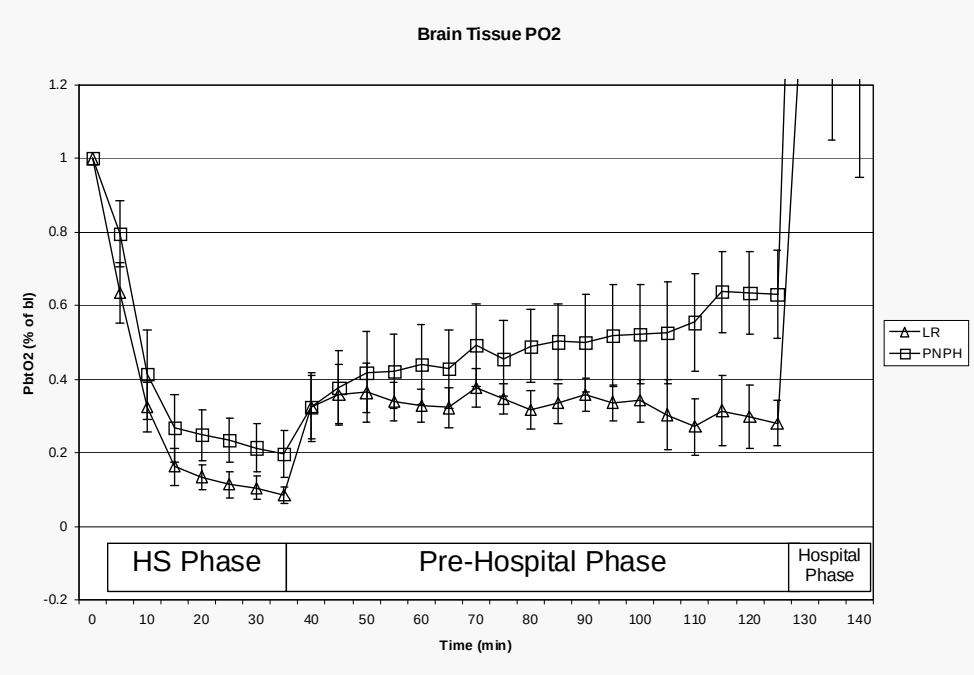

Figure 10. Effect of PNPH on recovery of hippocampal $PbtO_2$ after TBI+HS in mice. PNPH better normalized $PbtO_2$ during the final 30 min of resuscitation ($P<0.05$ vs LR).

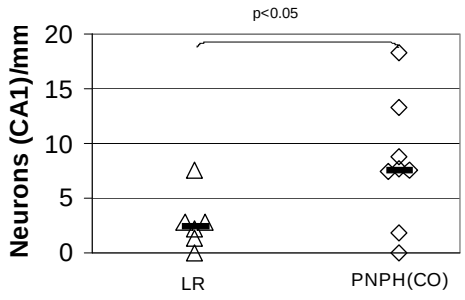

Figure 11. Hippocampal neuronal survival in CA1 at 24 h after resuscitation from TBI+HS in mice. PNPH significantly reduced neuronal death vs LR–confirming our prior findings with regard to neuropathology in study T4 .

<u>Study T7</u>. Effect of PNPH in the setting of TBI alone. Given its potent antioxidant properties and neuroprotective properties in both our *in vivo* and *in vitro* (see below) models of TBI, we have also carried out a complete study examining PNPH vs LR in the setting of TBI alone. In that study we administered a single bolus of PNPH vs LR early after the injury, followed acute physiology (MAP, blood gases, plasma Hb concentration) and 24 h neuropathology. MAP was nearly normalized early after TBI by PNPH vs mild hypotension with CCI alone (see **Figure 12**); neuropathology is being processed. **This study will be important to determine if PNPH is either beneficial in the setting of TBI alone—which would even further broaden its potential utility in TBI resuscitation. Our data strongly suggest a beneficial hemodynamic effect of PNPH even in the setting**

TBI alone. Data analysis of the neuropathology is ongoing and specifically includes FJC staining assessed at 24 h after the injury. This study was carried out by Clayton Lewis, a medical student at the University of Pittsburgh School of Medicine and is currently being prepared as a full manuscript (9).

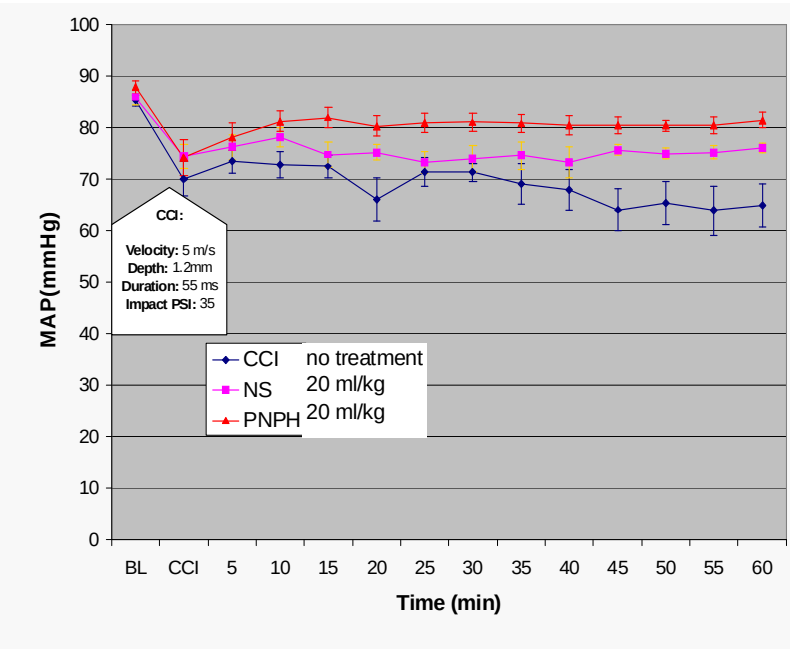

Figure 12. Study of the effect of PNPH vs LR in the setting of TBI alone without HS. Time course of MAP in mice after CCI treated with 1) no resuscitation fluid, 2) normal saline (20 mL/kg) or PNPH (20 mL/kg). Thus, PNPH exhibits a favorable hemodynamic profile even in the setting of TBI alone. Neuropathology is pending.

<u>Studies M5and M6</u>. *Assessment of brain edema and ICP after TBI+HS in mice*: We also assessed brain edema after resuscitation in LR treated mice subjected to combined TBI+HS using MRI methods (at 4.7 Tesla)—quantifying brain edema, regional CBF, and blood-brain barrier permeability. Data from our study of the effect of TBI+HS on brain edema by MRI is currently being analyzed and will represent a separate future publication. In that study, we compared LR, PNPH, and a recombinant octameric Hb (25, 28). We also explored another key facet of monitoring relevant to TBI+HS, namely ICP monitoring. We completed a small series of mice in which ICP monitoring was carried out in naïve, TBI alone, and TBI+HS using parenchymal placement of a 1-French Mylar catheter. Details of those studies are currently being analyzed, and although we did not feel that given the technical challenges in mice, ICP could represent a key outcome parameter in our studies--we believe that it is important to know–for the discussion section of our publications—what the time course and general magnitude of ICP changes are in our murine model.

<u>Study T8.</u> ***PNPH is a non-toxic Hb in vitro:*** We were concerned about the possibility of direct toxicitiy from PNPH to neurons—if extravasation of PNPH occurred into brain tissue after TBI resuscitation, since—as discussed—it is well known from the classic studies of Regan and Panter (*Neurosci Lett*, 1993; *J Neurotrauma*, 1993) that cell free Hb is neurotoxic in neuronal cell culture models. For example, in severe TBI, there is important blood-brain barrier disruption, and thus, if one were to resuscitate severe TBI victims in HS with PNPH, despite important benefits on cerebral hemodynamics, direct toxicity of the Hb moieties could be problematic. Thus, we

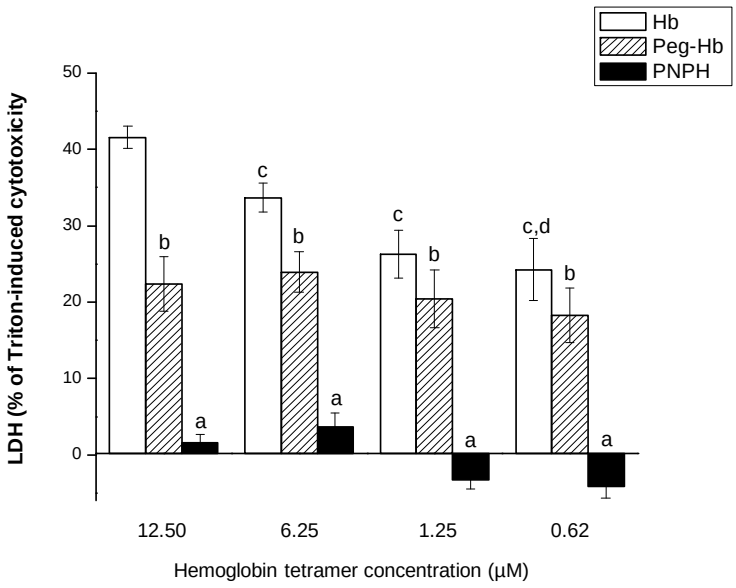

Figure 13. Effect of Hbs on LDH release from primary rat cortical neurons in culture. Hbs were added to the culture medium for 24 h at concentrations from 0.625-12.50 µM. Cytotoxicity (LDH release relative to Triton exposure) is graphed. Native bovine Hb (Hb) showed dose-dependent neurotoxicity. Polyethylene glycol (Peg) conjugated bovine Hb (Peg-Hb) showed less toxicity vs native Hb while PNPH was devoid of toxicity across concentrations. Data are mean+SEM. $^{a}p<0.05$ vs all Hb and Peg-Hb concentrations; $^{b}p<0.05$ vs Hb at the 12.50 and 6.25 µM; $^{c}p<0.05$ vs Hb at 12.50 µM; $^{d}p<0.05$ vs Hb at 6.25µM.

also carried out comprehensive *in vitro* studies evaluating PNPH in cultured rat primary neurons. We compared the effects of PNPH to two other logical control Hbs, namely, native bovine Hb (the base molecule for Peg-Hb), and bovine Peg-Hb (Prolong Pharmaceuticals), the specific parent molecule for PNPH. Recall that Peg-Hb is nitroxylated at ~14 sites to produce PNPH. First, unlike either control bovine Hb or bovine peg-Hb (which were neurotoxic), we observed a lack of toxicity of PNPH in rat primary neurons across a wide range of concentrations, with a marked reduction in neuronal death—reflected by two independent assays, 1) LDH release (**Figure 13**), and 2) MTT (**Figure 14**). As expected, native bovine cell free Hb was highly neurotoxic in a dose dependent fashion. In contrast, PNPH was totally devoid of neurotoxicity across the entire concentration range tested. Interestingly, Peg-Hb showed intermediate protection. This important finding indicated that PNPH may have special properties that are quite favorable for TBI resuscitation. These findings were presented at the 12th International Symposium on Blood Substitutes and the 2009 National Neurotrauma Society Congress (24, 26) and are in revision as a full manuscript (along with our in vivo work addressing neuroprotection) in the journal *Critical Care Medicine* (4). **We believe that this is another important finding supporting future development of PNPH.**

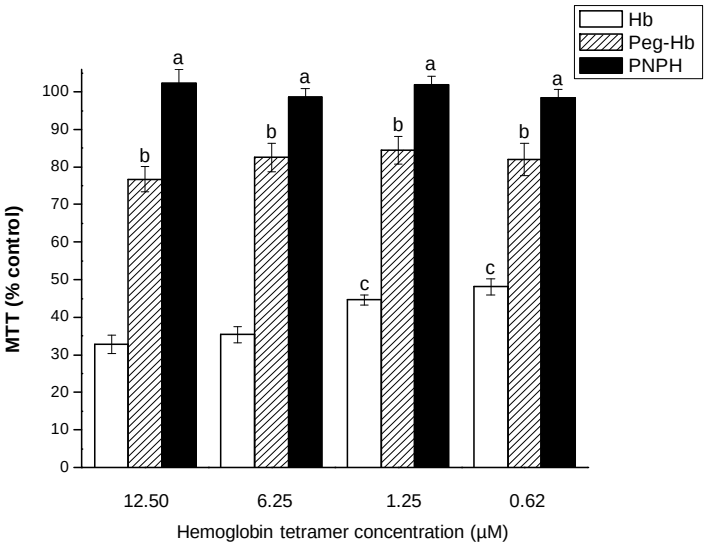

Figure 14. Effect of Hbs on cell viability accessed via the MTT assay in primary rat cortical neurons in culture. Hbs were added to the medium for 24h at 0.625-12.50 μM. MTT (% baseline) is shown. Native bovine Hb (Hb) showed dose-dependent toxicity. Polyethylene glycol-conjugated bovine Hb (Peg-Hb) showed less toxicity vs native Hb while PNPH was devoid of toxicity across concentrations. Data are mean+SEM. [a]$p<0.05$ vs all Hb and Peg-Hb at all concentrations; [b]$p<0.05$vs Hb at all concentrations; [c]$p<0.05$ vs Hb at 12.50 and 6.25 μM.

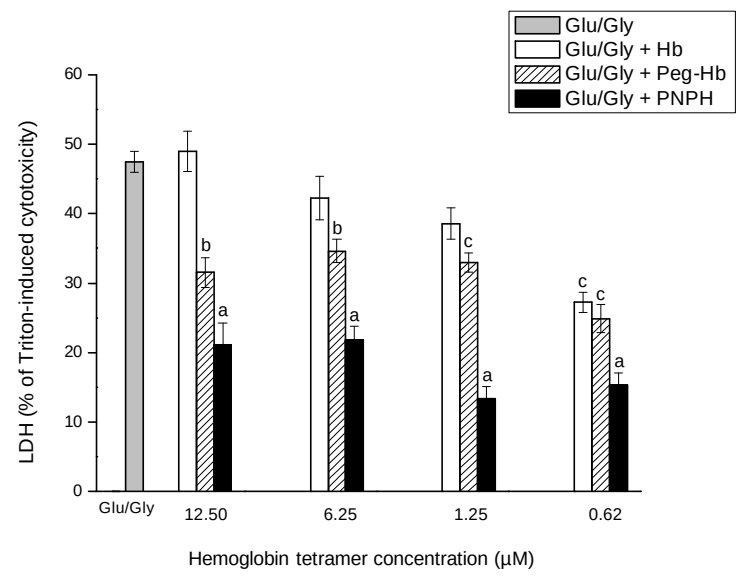

Figure 15. Effect of Hbs on LDH release in a primary rat cortical neuron culture model of glutamate/glycine (Glu/Gly)-induced excitotoxicity. The test Hb was added to the culture medium and 30 min later Glu/Gly (10 μM each) exposure was begun and continued for 24 h. Hb concentrations ranged from 0.625-12.50 μM. Neurotoxicity (LDH release relative to Triton exposure) is graphed. PNPH showed surprising neuroprotection at all concentrations. Peg-Hb showed intermediate protection, while native bovine Hb (Hb) was not protective. Data are mean+SEM. [a]$p<0.05$ vs Glu/Gly and both respective Hb and Peg-Hb; [b]$p<0.05$ vs Glu/Gly and respective Hb; [c]$p<0.05$ vs Glu/Gly.

<u>Study T9.</u> ***PNPH is neuroprotective against excitotoxic brain injury in vitro.*** Given the surprising lack of neurotoxicity of PNPH vs native cell free Hb in neuronal culture, and in light of the potent neuroprotection that we observed in our in vivo studies of TBI+HS in mice, it was logical to explore the possibility that PNPH might show direct neuroprotective effects in *in vitro* models of TBI. First, we studied the effect of PNPH in a classic model of excitotoxic neuronal death produced by exposure to glutamate and glycine. **The findings were remarkable. Unlike control**

bovine Hb, which at high concentrations exacerbated neuronal death, PNPH consistently attenuated neuronal death in culture as assessed using both LDH and MTT assays. These studies suggest direct neuroprotective effects of PNPH. Of note, once again Peg-Hb showed intermediate neuroprotection. Thus, unique properties of PNPH such as its antioxidant effects, along with beneficial effects of either the Peg moieties or the CO (both of which are common to both PNPH and Peg-Hb) appear to be acting synergistically to confer potent neuroprotection to PNPH. Oxidative stress is known to play an important role in excitotoxicty. That may, thus, represent the key way in which PNPH is showing direct neuroprotection. Currently, we are studying the mechanistic underpinnings for the neuroprotection by PNPH in our laboratory. These findings were also presented at the 12th International Symposium on Blood Substitutes and the 2009 National Neurotrauma Society Congress (24, 26) and are also part of the work in revision as a manuscript in the journal *Critical Care Medicine* (4). **We believe that these extremely novel and unique neuroprotective properties of PNPH also strongly support future development of PNPH.**

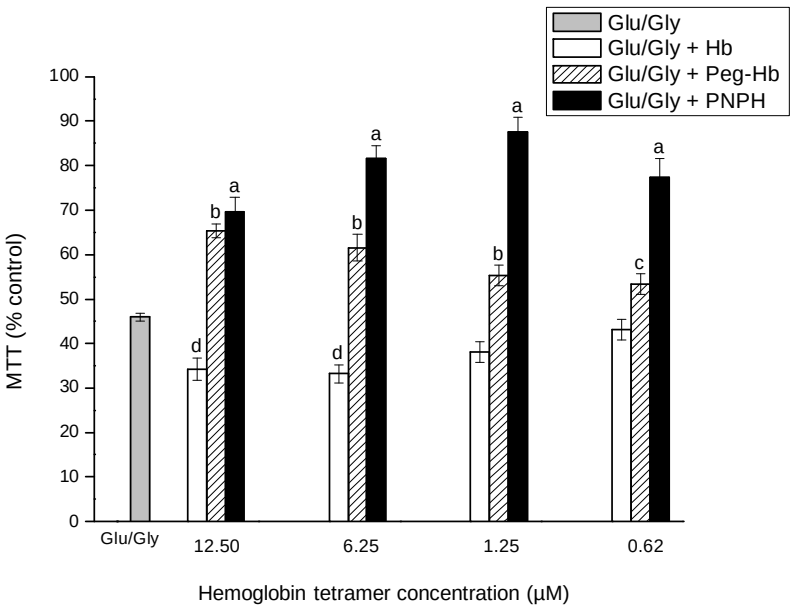

Figure 16. Effect of Hbs on cell viability (MTT assay) in a primary rat cortical neuron culture model of glutamate/glycine (Glu/Gly)-induced excitotoxicity. The test Hb was added to the culture medium and 30 min later Glu/Gly (10 μM) exposure was begun and continued for 24 h. Hb concentrations ranged from 0.625-12.50 μM. MTT (% baseline) is graphed. PNPH showed surprising neuroprotection at all concentrations. Peg-Hb showed intermediate protection, while native bovine Hb (Hb) exacerbated Glu/Gly toxicity at the highest concentrations. Data are mean+SEM. [a]$p<0.05$ vs Glu/Gly and both respective Hb and Peg-Hb; [b]$p<0.05$ vs Glu/Gly and respective Hb; [c]$p<0.05$ vs Glu/Gly.

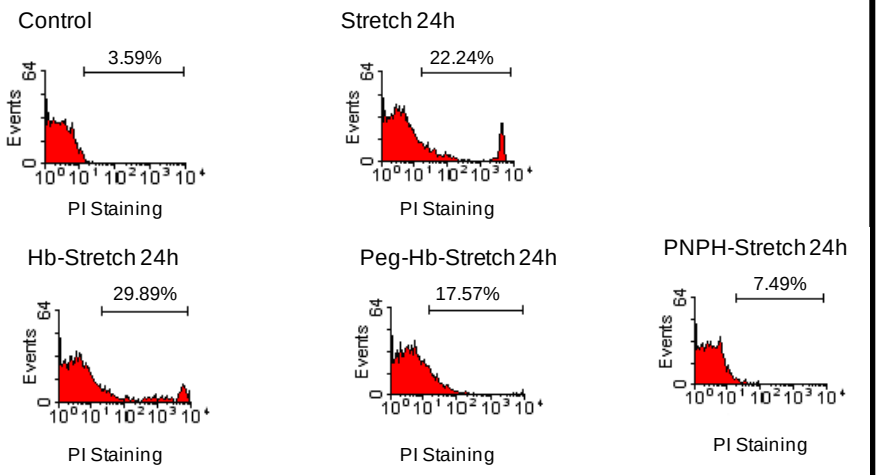

Figure 17. Effect of bovine Hb, Peg-Hb and PNPH (1.25 μM) on rat cortical neurons in culture exposed to *in vitro* trauma produced by neuronal stretch. Flow cytometric analysis of propidium iodide (PI)-labeled cells at 24 h showing increased PI staining in stretched vs. control neurons (22.2 vs. 3.6%, respectively). Neuronal death after stretch injury is exacerbated by Hb (29.9%), unaffected by Peg-Hb (17.6), and reduced by PNPH (7.5%).

<u>Study T10.</u> ***PNPH is neuroprotective against in vitro TBI produced by neuronal stretch.*** The effect of the three Hb preparations was also assessed in a neuronal stretch model at the 1.25μM concentration, with cell death assessed at 24 h quantitatively via flow cytometry using propidium iodide (PI) labeling (**Figure 17**), and qualitatively using microtubule-associated protein-2 (MAP-2) immunofluorescence (**Figure 18**). A computer controlled apparatus to stretch neurons at a defined strain magnitude and rate was used. Briefly, primary cortical neurons were grown on silicone membranes (0.002-0.005 inch thick, Specialty Manufacturing) secured to stainless steel rings that were polished and

passivated prior to use. At 8 days *in vitro*, cultures were pre-treated with varying concentrations of Hb for 30 min. The membranes were then placed over a hollowed platform in a custom-made, sealed stainless steel chamber. The membranes were then stretched with a pre-set strain rate (10 s^{-1}) and membrane deformation (50%) using an air pressure pulse. The pressure waveform is measured and collected on a data acquisition system to verify the degree of insult. Severe stretch was chosen in order to simulate a strain field similar to that seen in animal models of TBI. Neuronal cultures were then returned to the incubator. Cultures were pre-treated with varying concentrations of Hb for 30 min. In the stretch model, both analyses showed a reduction in neuronal death by PNPH, again with an intermediate effect of Peg-Hb and no benefit from bovine Hb. These data were also part of the aforementioned presentations (24, 26), and the manuscript currently in revision (4).

Taken together, the *in vivo* data showing neuroprotection and improved $PbtO_2$, and the exciting and unique data from *in vitro* experiments (T8-T10) showing neuroprotection, PNPH may represent an agent that produces a paradigm shift in potential utility of HBOCs in TBI resuscitation; namely, PNPH is a novel Hb that confers direct neuroprotective rather than neurotoxic effects. In addition, it is tempting to speculate that this cytoprotection could potentially be operating in any tissue in which there is hemorrhage or vascular disruption—and where an HBOC would be proposed for use (i.e., trauma resuscitation). If confirmed with additional studies, PNPH has the potential to produce a paradigm shift for the entire blood substitute field with regard to the cellular toxicity of Hb—and PNPH may thus represent a much less cytotoxic HBOC than a conventional non-nitroxylated and non-pegylated Hb. The data generated from this grant strongly support this hypothesis in TBI and this may reflect the fact that as discussed oxidative stress plays a key role in excitotoxicity, which is an established secondary injury mechanism in TBI. Beneficial effects of PNPH on other secondary injury mechanisms could also be important.

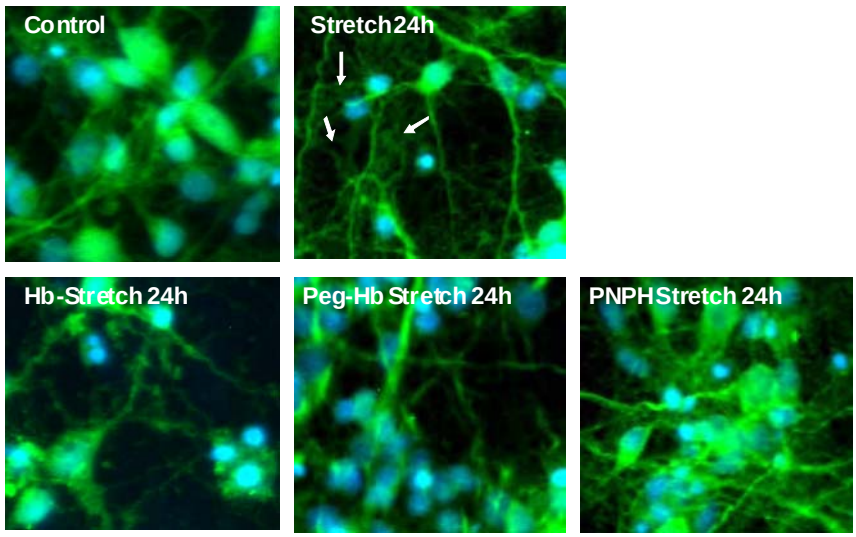

Figure 18. **E**ffect of bovine Hb, Peg-Hb and PNPH (1.25 µM) on rat cortical neurons in culture exposed to *in vitro* TBI produced by neuronal stretch. Microtubule-associated protein-2 (MAP-2) immunofluorescence (green) at 24 h showing loss of neurons and neurite damage after stretch injury. Unlike control bovine Hb or Peg-Hb, PNPH appears to reduce neuronal death. Nuclei were labeled with bisbenzimide (blue).

<u>Study M7.</u> ***Mechanistic studies of the effect of nitroxides on oxidative stress including use of oxidative lipidomics.*** Included in the studies evaluating the effect of these novel nitroxide containing colloids and Hb (i.e., PNA and PNPH) in our *in vivo* and *in vitro* models of TBI and TBI+HS, we assessed a battery of markers of oxidative stress including (for in vivo studies) glutathione, low molecular weight thiols, total antioxidant reserve, and oxidative lipidomics. In addition, our collaborative team at the Pittsburgh Center for Free Radical and Antioxidant Health (Drs. Bayır and Kagan). In year 4, based on the exciting in vitro findings showing unique neuroprotection of PNPH in vitro, effects of PNPH on oxidative stress in vitro were also explored. Details of these studies were provided in prior reports. A manuscript on in vivo application of the oxidative lipidomics technique to studying oxidative injury to mitochondrial lipids in the CCI model of TBI was published as previously described (4). **These data were presented in prior reports and are not presented in lieu of the full publication of this work (4).** A report describing in vitro effects of PNPH on oxidative stress in our cell culture model is also in preparation.

Study M8. ***Assessment of functional outcome deficits after combined TBI plus HS in mice:*** We also characterized functional outcome in our more severe pressure controlled version of combined TBI plus HS. Given that improvement in behavioral outcome will ultimately be needed to move PNPH to achieving an IND for a new agent to be used for TBI resuscitation, we carried out a full characterization of functional outcome and 21 day neuropathology in the model. Morris water maze (MWM) latency to find the between 14 and 21 days after injury was the primary outcome for these studies using a traditional spatial memory acquisition paradigm (hidden platform). These studies were carried out in collaboration with Dr. C. Edward Dixon, an authority on functional outcome testing in experimental TBI models. **Despite using a mild CCI, a level that in and of itself produces no significant MWM deficit in our laboratory, combined CCI plus 35 min of severe HS (MAP=25 mmHg)—showed an obvious impairment (~doubling of latency to find the hidden platform) in functional outcome that can readily serve as the primary outcome for future studies to bring PNPH to IND (Figure 19). In addition, we will be carrying out studies on severe DOD-related grants (DARPA PREVENT II, a consortium grant under the direction of Dr. James Atkins at WRAIR, and a multi-center drug screening grant titled Operation Brain Trauma Therapy funded by the US Army. Thus, this new model will be perfect for future testing of novel therapies for blast TBI/polytrauma in these DOD-supported initiatives. This work was carried out in part by University of Pittsburgh Medical Student Joseph Hemerka and will be submitted to the 2010 meeting of the National Neurotrauma Society as an abstract and is in preparation as a full manuscript (10).**

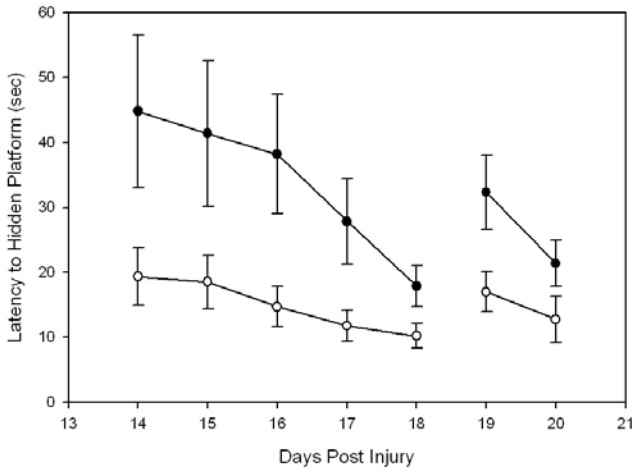

Figure 19. Functional outcome in mice in a spatial memory acquisition paradigm in the Morris water maze. Latency to find the hidden platform was prolonged during testing on d 14-18 after combined TBI+HS using the pressure controlled HS model described in Figure 8 (solid circles) vs sham (open circles). Visible platform testing was performed to rule out nonspecific deficits on d 19-20. These studies show utility of this model for future studies to acquire an IND for PNPH and for screening other potential therapies for TBI resuscitation in combat casualty care (see text for details).

KEY RESEARCH ACCOMPLISHMENTS

1. Established the first experimental model of TBI+HS in mice. This was a volume controlled HS model with 90 min of HS (~30% blood volume with MAP of ~35-40 mmHg) superimposed upon TBI produced by relatively mild CCI (1 mm depth and 5 m/sec velocity).
2. Established a second more severe experimental model of TBI+HS in mice. This was a pressure controlled HS model with 35 min of severe HS (MAP clamped at 25-27 mmHg) superimposed upon TBI produced again by relatively mild CCI.
3. Serial non-invasive MRI assessment of CBF in the volume controlled HS model of TBI+HS demonstrating that exacerbation of brain damage in hippocampus between 60 and 90 min of HS is associated with deterioration of CBF in the hippocampus.
4. Demonstration in both our murine volume controlled and pressure controlled TBI+HS models that the novel colloid PNA or the current standard of care agent in combat casualty care HEX represent small volume resuscitation solution that have favorable effects on MAP vs LR or hypertonic saline. However despite these favorable properties, neither PNA nor HEX were neuroprotective in the TBI+HS model.

5. Demonstration in both our volume controlled and pressure controlled TBI+HS models that the novel HBOC PNPH represents an extremely small volume resuscitation solution that has a favorable effect on MAP vs LR or HEX and that this is also accompanied by favorable effects on blood lactate and pH.
6. Demonstration in both our volume controlled and pressure controlled TBI+HS models that the novel HBOC PNPH confers neuroprotective effects – ameliorating hippocampal neuronal death at both 24 h and 7 d after resuscitation.
7. Establishment of $PbtO_2$ and ICP monitoring in our mouse models of combined TBI+HS.
8. Demonstration in our pressure controlled model of TBI+HS that PNPH improves $PbtO_2$ vs LR when used for 90 min during resuscitation.
9. Demonstration in an in vitro primary neuronal culture system that PNPH is unique non-neurotoxic, unlike either native bovine Hb or Peg-Hb (which showed intermediate toxicity).
10. Demonstration in an in vitro primary neuronal culture model of in vitro TBI produced by neuronal stretch that PNPH is unique neuroprotective Hb, unlike either native bovine Hb (which exacerbates toxicity) or Peg-Hb (which showed intermediate toxicity).
11. Use of standard and novel markers of oxidative stress to assess the effect of secondary HS after TBI on oxidative stress and study the role of the nitroxide moieties in PNPH *in vivo* and *in vitro*. This included development of oxidative lipidomics methods for *in vivo* application in TBI and TBI+HS, specifically showing early selective oxidation of the mitochondrial lipid, cardiolipin which is linked to apoptosis.
12. Characterization of functional outcome (assessed by Morris water maze testing) in our combined TBI+HS model in mice, which should be extremely useful to take PNPH to IND and for a number of newly funded initiatives for the US Army and DARPA to screen novel therapies for blast TBI and polytrauma.

REPORTABLE OUTCOMES

1. Publication of our first paper supported by this grant (1) titled "Hemorrhagic Shock after Experimental Traumatic Brain Injury in Mice: Effect on Neuronal Death," describing our mouse model of TBI+HS in the June 2009 issue of the *Journal of Neurotrauma*. This model was very useful for studying the effects of both PNA and PNPH in TBI resuscitation as described in this report. It will also be very useful for future DOD funded studies to screen new potential therapies for TBI resuscitation in combat casualty care.
2. Publication of a second paper supported by this grant (2) titled "Resuscitation of Traumatic Brain Injury and Hemorrhagic Shock with Polynitroxyl Albumin: Effects on Acute Hemodynamics, Survival, and Hippocampal Histology," in the Dec 2009 issue of the *Journal of Neurotrauma* describing the efficacy of the novel compound polynitroxylated albumin (PNA) as a small volume resuscitation solution with similar efficacy as HEX and a favorable profile vs either LR or HS in our experimental model of TBI+HS. However, none of these solutions (including PNA) conferred neuroprotection against neuronal death in the CA1 or CA3 regions of the hippocampus, which are important targets for TBI victims.
3. Publication of a third paper supported by this grant describing successful development and application of a novel oxidative lipidomics method to assess mitochondrial oxidative stress including early, selective oxidation of the mitochondrial lipid cardiolipin after experimental TBI in rats. That work was titled "Selective Early Cardiolipin Oxidation after Brain Trauma: A Lipidomics Analysis," and is published as a full paper in *Annals of Neurology* (3). It is already a highly cited paper.
4. Preparation, submission, revision and re-submission of our fourth paper supported by this grant (4) titled "Polynitroxylated Pegylated Hemoglobin: A Novel Neuroprotective Hemoglobin for Acute Volume-Limited Fluid Resuscitation after Combined Traumatic Brain Injury and Hemorrhagic Hypotension in Mice" to the journal *Critical Care Medicine.* This manuscript describes both our in *vivo* and *in vitro* work showing unique neuroprotection of the novel HBOC PNPH both in our mouse model of TBI+HS and in three *in vitro* model systems, namely, 1) primary rat neuronal culture, 2) glutamate/glycine-induced neuronal death in culture, and 3) neuronal stretch injury in culture. We also reported that *in*

vivo, PNPH acts as a small volume resuscitation solution, but as indicated it has unique neuroprotective properties, unlike PNA, HEX, LR, or hypertonic saline. In the *in vitro* studies, PNPH was compared to the parent unmodified bovine Hb which was actually neurotoxic, and pegylated (Peg) Hb (Peg-Hb), which showed intermediate neuroprotection. **We believe that these findings are important to the blood substitute, TBI, and resuscitation fields, and represent a seminal finding.**

5. Demonstration that PNPH improves $PbtO_2$ vs LR in experimental TBI+HS resuscitation and presentation of that work at two international meetings (26, 29).
6. Demonstration that PNPH also improves hemodynamics in the setting of experimental TBI without HS—a finding which would potentially allow safe use of PNPH in the broader application of TBI resuscitation—when hypotension after injury may be present even without obvious hemorrhage.
7. Establishment of an in vitro neuronal stretch model using primary neuron culture in our center for study of in vitro TBI. This model was used to confirm the unique direct neuroprotective effects will be used in our future DOD supported work to aid in screening drugs for potential neuroprotection in TBI for combat casualty care.
8. Establishment and complete characterization including hemodynamic and $PbtO_2$ monitoring, functional outcome testing (Morris water maze), and long-term neuropathology of a severe pressure controlled HS model of combined TBI+HS in mice. This model was very useful for studying the effects of PNPH in TBI resuscitation as described in this report. It will also be very useful for future DOD funded studies to screen new potential therapies for TBI resuscitation in combat casualty care.
9. A total of 20 national and international presentations of this work were made including plenary presentations at ATACCC, the National Neurotrauma Society and to the NIH (18, 20, 30).
10. A total of 8 trainees worked on this project, including three fellows, a resident, a PhD candidate, and three medical students. One of the trainees received the In Training Award from the *SCCM*, one received a Scientific Award from the SCCM, and one trainee was an officer in the US Navy.

Please note that Co-investigators Drs. Li Ma and Carleton Hsia at SynZyme Technologies, have synthesized and provided PNA and PNPH for all of the studies that were carried out over the 4 years of funding. Please see report from work by Synzyme Technologies.

CONCLUSIONS

Taken together, our exciting and considerable work accomplished in this 4-year funding period suggest that initial pre-hospital resuscitation with PNPH could have significant and unique benefit to soldiers and civilians with TBI who experience hypotension and/or HS. Our data suggest that PNPH is a very unique and exciting neuroprotective Hb that may represent a paradigm shift in the blood substitute field. It appears to have very great potential in TBI resuscitation. We are, thus, poised to develop further PNPH as a therapeutic drug candidate moving forward to an IND for TBI resuscitation in both combat casualty care and civilian TBI.

Manuscripts resulting supported by this funding

1. Dennis AM, Haselkorn L, Vagni VA, Garman R, Janesko-Feldman K, Bayır H, Clark RSB, Jenkins LW, Dixon CE, Kochanek PM: Hemorrhagic shock after experimental traumatic brain injury in mice: Effect on neuronal death. J Neurotrauma 26:889-899, 2009.
2. Exo J, Shellington D, Bayır H, Vagni V, Feldman K, Ma L, Hsia C, Clark RSB, Jekins LW, Dixon CE, Kochanek PM: Resuscitation of traumatic brain injury and hemorrhagic shock with polynitroxyl albumin: effects on acute hemodynamics, survival, and hippocampal histology. J Neurotrauma 26:2403-2408, 2009.

3. Bayır H, Tyurin VA, Tyurin YY, Viener R, Ritov V, Amoscato A, Zhao Q, Zhang X, Feldman-Janesko KL, Alexander H, Clark RSB, Kochanek PM, Kagan VE: Selective Early Cardiolipin Oxidation after Brain Trauma: A Lipidomics Analysis. Ann Neurol 62:154-169, 2007.
4. Shellington DK, Du L, Wu X, Exo J, Vagni V, Ma L, Janesko-Feldman K, Clark RSB, Bayır H, Dixon CE, Jenkins LW, Hsia CJC, Kochanek PM: Polynitroxylated Pegylated Hemoglobin: A Novel Neuroprotective Hemoglobin for Acute Volume-Limited Fluid Resuscitation after Combined Traumatic Brain Injury and Hemorrhagic Hypotension in Mice. Crit Care Med (in submission 10/23/09; in revision 2/03/10)
5. Kochanek PM, Bauman R, Long J, Dixon CE, Jenkins LW: Blast-induced Traumatic Brain Injury and Polytrauma: A critical problem begging for new insight and new therapies. Foreword, *Special Issue on Blast-induced Traumatic Brain Injury*, J Neurotrauma 26:813-814, 2009.

Articles in preparation

6. Foley LM, Dennis AM, Hitchens TK, Melick JM, Wisniewski S, Ho C, Jenkins LW, Kochanek PM: MRI assessment of cerebral blood flow following experimental traumatic brain injury combined with hemorrhagic shock. (in preparation)
7. Wu X, Ho NT, Shellington D, Ho C, Vagni V, Simplaceanu V, Shen TJ, Kochanek PM: Recombinant hemoglobins as resuscitation fluids in a mouse model of traumatic brain injury plus hemorrhagic shock. (in preparation)
8. Wu X, Shellington DK, Ma L, Exo J, Vagni V, Janesko-Feldman K, Jenkins LW, Clark RSB, Bayır H, Hisa C, Kochanek PM: Resuscitation with the novel hemoglobin PNPH improves brain tissue oxygenation after combined experimental traumatic brain injury and hemorrhagic shock in mice. (in preparation)
9. Lewis C, Exo J, Shellington DK, Janesko-Feldman K, Ma L, Hsia C, Bayır H, Jenkins JW, Dixon CE, Clark RSB, Kochanek PM. Effect of administration of the novel hemoglobin PNPH after controlled cortical impact in mice. (in preparation)
10. Hemerka J, Dixon CE, Garman R, Janesko-Feldman K, Exo J, Shellington DK, Jenkins JW, Bayır H, Clark RSB, Kochanek PM: Severe pressure controlled hemorrhagic shock after experimental traumatic brain injury in mice: Effects on functional outcome and long-term neuropathology (in preparation).

Abstracts supported by this funding.

11. Dennis A, Vagni V, Feldman K, Clark RS, Jenkins L, Dixon CE, Bayır H, Kochanek PM: Exacerbation of neuronal injury by hemorrhagic shock after mild controlled cortical impact in mice. J Neurotrauma 23:1001, 2006.
12. Dennis AM, Foley LM, Melick JA, Hitchens TK, Jenkins LW, Clark RS, Ho C, Kochanek PM: Effect of hemorrhagic shock on cerebral blood flow in experimental traumatic braininjury: Magnetic resonance imaging assessment. SCCM 2007 Congress, Crit Care Med 34:A5, 2006.
13. Haselkorn ML, Dennis AM, Vagni V, Janesko-Feldman K, Clark RS, Jenkins LW, Dixon CE, Kochanek PM: Effect of hemorrhagic shock on neuronal death after experimental traumatic brain injury in mice. SCCM 2007 Congress, Crit Care Med 34:A17, 2006.

14. Foley LM, Dennis AM, Hitchens TK, Melick JA, Ho C, Kochanek PM: ASL-MRI assessment of the effect of hemorrhagic shock on cerebral blood flow after experimental traumatic brain injury in mice. ISMRM 15th Conference, Berlin, Germany, May 21-25, 2007, Abstract #2373.
15. Dennis AM, Haselkorn L, Foley LM, Garman R, Jenkins LW, Hitchens TK, Clark RS, Ho C, Bayır H, Kochanek PM: Effect of hemorrhagic shock on neuronal death and cerebral blood flow after experimental traumatic brain injury in mice: Magnetic resonance imaging assessment. The 25th Annual National Neurotrauma Society Symposium, J Neurotrauma 24:1237, 2007.
16. Exo J, Shellington DK, Vagni V, Feldman K, Ma L, Hsia C, Clark RS, Bayır H, Jenkins LW, Dixon CE, Kochanek PM: Nitroxide-based resuscitation of combined traumatic brain injury and hemorrhagic shock: effect on acute hemodynamics. Crit Care Med 35:A6, 2007.
17. Shellington DK, Exo J, Feldman K, vagni V, Clark R, Bell MJ, Jenkins L, Kochanek PM: Effect of hemorrhagic shock on the microglial response after traumatic brain injury in mice. Crit Care Med 35:A40, 2007.
18. Kochanek PM, Clark RS, Dixon CE, Garman R, Bayır H, Kagan VE, Jackson EK, Bauman R, Long J, Parks S, Shellington D, Exo J, Jenkins LW: Emergent Potential Treatments for Blast-Induced Neurotrauma. National Institute of Neurological Disorders and Stroke, Neurological Effects of Blast Injury Workshop, Rockville, Maryland, April 10-11, 2008.
19. Exo J, Shellington D, Vagni V, Feldman K, Li Ma L, Hsia C, Clark RSB, Bayır H, Jenkins L, Dixon CE, Kochanek PM: Resuscitation of Combined Traumatic Brain Injury and Hemorrhagic Shock with Polynitroxyl Albumin: Effect on Fluid Requirements, Blood Pressure, Survival and Neuropathology. J Neurotrauma 25:888, 2008.
20. Kochanek PM, Clark RSB, Dixon CE, Garman R, Bayır H, Kagan V, Jackson EK, Bauman R, Long J, Parks S, Jenkins L: Pathophysiology Guided Therapeutic Approaches To Blast-TBI and Polytrauma. The 26th Annual National Neurotrauma Symposium July 27-30, 2008.
21. Shellington DK, Exo J, Vagni V, Ma L, Feldman K, Clark RSB, Bayır H, Dixon CE, Jenkins LW, Abuchowski A, Hsia C, Kochanek PM: Polynitroxylated Pegylated Hemoglobin Solution for the Acute Limited Fluid Resuscitation of Hemorrhagic Shock after Traumatic Brain Injury in a Mouse Model. J Neurotrauma 25:868, 2008.
22. Shellington D, Wu X, Exo J, Vagni V, Feldman K, Clark R, Bayır H, Dixon CE, Jenkins L, Kochanek P, Ma L, Hsia C, Abuchowski A: Polynitroxylated pegylated hemoglobin for the acute limited fluid resuscitation of hemorrhagic shock after traumatic brain injury in a mouse model. 38th Critical Care Congress, January 31-February 4, 2009, Nashville, TN Crit Care Med 36(Suppl):A5, 2008.
23. Exo J, Shellington D, Vagni V, Feldman K, Ma L, Hsia C, Clark RSB, Bayır H, Jenkins L, Dixon CE, Kochanek PM: Resuscitation of traumatic brain injury and hemorrhagic shock with novel colloids: Effects on acute hemodynamics and survival. 38th Critical Care Congress, January 31-February 4, 2009, Nashville, TN Crit Care Med 36(Suppl):A144, 2008.
24. Kochanek PM, Wu X; Ma L, Du L, Shellington D; Vagni V, Clark RSB, Hsia C: PNPH, A neuroprotectant HBOC: Studies of in vivo and in vitro traumatic brain injury. XII Symposium on Blood Substitutes, Parma, Italy, August 25-28, 2009.
25. Wu X, Ho NT, Shellington D, Ho C, Vagni V, Simplaceanu V, Shen TJ, Kochanek PM: Recombinant hemoglobins as resuscitation fluids in a mouse model of traumatic brain injury plus hemorrhagic shock. The 2nd Joint Symposium of the International and National Neurotrauma Societies, Santa Barbara, California, September 7-11, 2009.

26. Wu X, Du L, Ma L, Shellington DK, Vagni V, Clark RSB, Bayır H, Hsia C, Kochanek PM: PNPH, A neuroprotectant HBOC: Studies of in vivo and in vitro traumatic brain injury. The 2nd Joint Symposium of the International and National Neurotrauma Societies, Santa Barbara, California, September 7-11, 2009.
27. Exo J, Shellington D, Vagni V, Feldman K, Ma L, Hsia C, Clark RSB, Bayır H, Dixon CE, Jenkins L, Kochanek P: Resuscitation of traumatic brain injury and hemorrhagic shock with crystalloid and colloid therapies: effects on acute resuscitation parameters, survival, and neuronal death. The 2nd Joint Symposium of the International and National Neurotrauma Societies, Santa Barbara, California, September 7-11, 2009.
28. Wu X, Ho N, Shellington DK, Ho C, Kochanek PM: Novel Recombinant Hemoglobins (rHb) for Traumatic Brain Injury Resuscitation in Mice. ASA Annual Meeting, October 17-21, 2009, New Orleans, Louisiana.
29. Wu X, Du L, Ma L, Shellington DK, Kochanek PM: Polynitroxylated Pegylated Hb (PNPH) in Resuscitation of Traumatic Brain Injury + Hemorrhagic Shock. ASA Annual Meeting, October 17-21, 2009, New Orleans, Louisiana.
30. Kochanek PM: Combat Casualty Care-relevant Studies at the Safar Center. ATACCC, St. Pete's Beach, Florida, August 10-12, 2009.

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APPENDICES

Separate PDF files are attached of each of the manuscripts or abstracts published that were supported in full or part by funding in year three of this program project grant.

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Hemorrhagic Shock after Experimental Traumatic Brain Injury in Mice: Effect on Neuronal Death

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Abstract

Traumatic brain injury (TBI) from blast injury is often complicated by hemorrhagic shock (HS) in victims of terrorist attacks. Most studies of HS after experimental TBI have focused on intracranial pressure; few have explored the effect of HS on neuronal death after TBI, and none have been done in mice. We hypothesized that neuronal death in CA1 hippocampus would be exacerbated by HS after experimental TBI. C57BL6J male mice were anesthetized with isoflurane, mean arterial blood pressure (MAP) was monitored, and controlled cortical impact (CCI) delivered to the left parietal cortex followed by continued anesthesia (CCI-only), or either 60 or 90 min of volume-controlled HS. Parallel 60- or 90-min HS-only groups were also studied. After HS (±CCI), 6% hetastarch was used targeting MAP of ≥50 mm Hg during a 30-min *Pre-Hospital* resuscitation phase. Then, shed blood was re-infused, and hetastarch was given targeting MAP of ≥60 mm Hg during a 30-min *Definitive Care* phase. Neurological injury was evaluated at 24 h (fluorojade C) or 7 days (CA1 and CA3 hippocampal neuron counts). HS reduced MAP to 30–40 mm Hg in all groups, $p < 0.05$ versus CCI-only. Ipsilateral CA1 neuron counts in the 90-min CCI+HS group were reduced at 16.5 ± 14.1 versus 30.8 ± 6.8, 32.3 ± 7.6, 30.6 ± 2.2, 28.1 ± 2.2 neurons/100 μm in CCI-only, 60-min HS-only, 90-min HS-only, and 60-min CCI+HS, respectively, all $p < 0.05$. CA3 neuron counts did not differ between groups. Fluorojade C staining confirmed neurodegeneration in CA1 in the 90-min CCI+HS group. Our data suggest a critical time window for exacerbation of neuronal death by HS after CCI and may have implications for blast injury victims in austere environments where definitive management is delayed.

Key words: blast; controlled cortical impact; delayed neuronal death; hippocampus; hypotension; mouse; polytrauma; resuscitation; secondary insult; selective vulnerability

Introduction

The important role of secondary insults in increasing morbidity and mortality after traumatic brain injury (TBI) is widely recognized, both experimentally and clinically. The combination of TBI and hemorrhagic shock (HS) has taken on special importance in both military and civilian settings as the result of terrorist attacks with improvised explosive devices, which inflict TBI and other extracerebral injuries (Gawande, 2004; Gutierrez de Ceballos et al., 2005). The report of Chesnut et al. (1993), reviewing the NIH Traumatic Coma Databank, correlated hypotension and hypoxemia with doubled morbidity and mortality after TBI in humans, identifying hypotension as the single most critical parameter. Subsequent work has confirmed the critical detrimental role of secondary insults after TBI in the intensive care unit (ICU) (Gopinath et al., 1994). The marked deleterious effects of secondary insults have been confirmed in the setting of blast polytrauma (Nelson et al., 2006). Early reports of exacerbation of brain injury by secondary insults in experimental TBI included work in the cats by Nelson et al. (1979), Jenkins et al. (1986), and Barron et al, (1988), and after fluid percussion injury (FPI) in rats by Ishige et al. (1987a,b), where brief periods of hypoxemia were used. Exacerbation of hippocampal neuronal death in the CA3 region by a secondary hypoxic insult was later observed by Clark et al. (1997) using a hypoxic admixture to achieve a PaO_2 of ~40 mm Hg after controlled cortical impact (CCI), and ex-

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acerbated hippocampal neuronal death in CA1 was reported by Jenkins et al. (1989) with transient carotid occlusion and hemorrhagic hypotension (producing forebrain ischemia) after FPI in rats. In rodent models of experimental TBI alone, neurons in the CA3 and hilar sectors of the hippocampus generally exhibit the greatest vulnerability (Lowenstein et al., 1992), while pyramidal neurons in the CA1 sector of the hippocampus are classically selectively vulnerable to ischemia or hypoxemia (Kirino, 1982). Both CA1 and CA3 hippocampal neuronal death are often identified on post-mortem examination after fatal TBI in humans (Kotapka et al., 1994). And these patients typically had secondary insults.

The post-TBI milieu is characterized by primary injury, cascades of secondary injury and repair, and less well recognized perturbations of normal homeostatic mechanisms. Considerable evidence supports the existence of marked vulnerability of the traumatically injured brain early after insult. A number of mechanisms are proposed to mediate this enhanced vulnerability including hypoperfusion and reduced oxygen delivery, disturbed autoregulation of cerebral blood flow (CBF), excitotoxicity, and mitochondrial failure, among others (DeWitt et al., 1995). These mechanisms may create an environment in the acutely injured brain that renders it vulnerable to a level of hypotension and anemia from HS that would otherwise be tolerated. In addition, secondary injury mechanisms during resuscitation and reperfusion may further exacerbate the evolution of damage. Given that HS produces maximal vasoconstriction in the splanchnic circulation (while the brain is relatively protected), visceral ischemia with resultant release of pro-inflammatory mediators and/or translocation of intestinal flora may also play a role in amplifying secondary brain damage (Vatner, 1974; Myers et al., 1994). This complex secondary injury cascade in the setting of combined TBI and HS is poorly understood.

Previous experimental models of combined TBI and HS have focused on hemodynamics and the effect of fluid resuscitation on intracranial pressure (ICP) and related intracranial dynamics using large animals (DeWitt et al., 1992a,b; Glass et al., 1999). Few studies have explored the effect of HS on neuronal death mechanisms after TBI in rodents (Matsushita et al., 2001), but none, to our knowledge, has specifically examined the impact of HS on hippocampal neuronal death in selectively vulnerable brain regions. There has also been a paucity of investigation of combined TBI and secondary insults in mice, a species ideal for mechanistic and therapeutic investigation due to ready availability of targeted mutant strains.

We report the characterization of a clinically relevant mouse model of combined TBI and HS, and resuscitation including physiologic monitoring and neuropathologic evaluation. We hypothesized that a level of HS that alone produces no neuronal damage (Carrillo et al., 1998) would increase neuronal death after experimental TBI in the CA1 ischemia-vulnerable region of the hippocampus.

Methods

Study groups and experimental protocol

The Institutional Animal Care and Use Committee of the University of Pittsburgh School of Medicine approved all experiments. Male C57BL6J mice (Jackson Laboratories, Bar Harbor, ME), 12–15 weeks of age and weighing 27 ± 1.8 g, were housed under controlled environmental conditions and allowed ad libitum food and water until study.

Anesthesia was induced with 4% isoflurane in oxygen and maintained with 1% isoflurane in 2:1 N_2O/O_2 via nose cone. Inguinal cut-down and insertion of central femoral venous and arterial catheters was accomplished under sterile conditions using modified polyethylene (PE)–50 tubing. After placement of the mouse in a stereotaxic frame, a 5-mm craniotomy was performed over the left parietotemporal cortex with a dental drill, and the bone flap was removed. A brain temperature micro-probe (Physitemp, Clifton, NJ) was inserted through a left frontal burr hole, and a rectal probe placed to monitor body temperature. Immediately after craniotomy, the inhalational anesthesia was changed to 1% isoflurane and room air for a 10-min equilibration period prior to beginning the injury protocols.

While brain temperature was maintained at 37 ± 0.5°C, mild-moderate CCI was performed with a pneumatic impactor (Bimba, Monee, IL) as previously reported with modifications (Sinz et al., 1999; Whalen et al., 1999). A 3-mm flat-tip impounder was deployed at a velocity of 5 m/sec and a depth of 1 mm. This injury level for CCI was specifically chosen to produce a contusion but no appreciable loss of hippocampal neurons in any subfield, in the absence of HS, based on prior work with this model in mice by our group (Kochanek et al., 2006; Foley et al., 2008), along with additional pilot studies.

A diagram of the experimental paradigm for TBI, shock, and resuscitation is provided in Figure 1. To model a level of HS that was clinically relevant, we performed a series of pilot experiments to determine the amount of hemorrhage volume necessary to reduce the mean arterial blood pressure (MAP) to achieve a stable MAP of ~35–40 mm Hg in all groups. Based on prior studies, we did not anticipate that this level of HS alone would produce brain injury (Carillo et al., 1998). Similarly, based on prior studies of the effect of HS on MAP in rodents with or without TBI (Yuan and Wade, 1992), and by a series of pilot experiments, it was determined that in mice subjected to HS alone, a volume of 2.7 mL/100 g was needed to achieve this target MAP range. In contrast, but as anticipated, after CCI, a smaller volume of 2.0/100 g was required, consistent with the well-described enhanced sensitivity to the hypotensive effects of hemorrhage after TBI (Yuan and Wade, 1992; Law et al., 1996). In all mice, HS was induced over 15 min in a decelerating fashion, with 50% of the total volume removed over the first 5 min, 25% over the next 5 min, and the final 25% over the last 5 min. Mice remained in unresuscitated HS for an additional 45 or 75 min for a total *Shock* phase of either 60 or 90 min, to study the effect of HS duration on neuropathological outcome after CCI. After completion of the blood withdrawal, mice transiently auto-resuscitated to a MAP of ~45–55 mm Hg, but then rapidly re-equilibrated and maintained MAP in the target range for the remainder of the desired 60–90-min *Shock* phase. After completion of the HS interval, a 30-min *Pre-Hospital* phase was initiated, and 6% hetastarch (Hextend, Hospira, INC., Lake Forest, IL) was rapidly infused in 0.1-mL aliquots to achieve a MAP of ≥50 mm Hg. To simulate arrival at more *Definitive Care*, mice were then switched from 1% isoflurane in room air to 1% isoflurane in oxygen. For this 30-min interval, shed blood was first rapidly re-infused, and a goal MAP of ≥60 mm Hg was maintained with addi-

tional 6% hetastarch, again administered in 0.1-mL aliquots. At completion of the *Definitive Care* phase, catheters were removed, anesthesia discontinued, and mice recovered in supplemental oxygen for 30 min before being returned to their cages.

Mice were randomized to one of five study groups (n = 10 per group), and underwent procedures or equivalent anesthesia and monitoring as designated: (1) CCI-only, (2) 60 min of HS only [60HS-only], (3) 90 min of HS-only [90HS only], (4) CCI followed immediately by 60 min of HS [60CCI+HS], or (5) CCI followed immediately by 90 min of HS [90CCI+HS]. Mice in the CCI-only group underwent CCI without HS, but were maintained under identical anesthesia and monitoring to the combined injury groups for a 60-min interval. Mice in the HS-only group underwent either 60 or 90 min of HS without craniotomy or CCI, but again were maintained under identical anesthesia and monitoring as the combined injury groups. Mice in the CCI+HS groups underwent CCI followed by HS of either 60 or 90 min duration as described above.

Monitoring protocol

MAP was continuously monitored via the femoral artery and recorded at baseline, after CCI, and every 5 min during HS and resuscitation; heart rate was continuously monitored and recorded at baseline and once during each phase. Laboratory evaluation with arterial blood gas determinations, and blood lactate, glucose, hematocrit, sodium, potassium, ionized calcium, and ionized magnesium was obtained at baseline, 30 min into the *Shock* phase, and at the end of the *Definitive Care* phase.

Histology protocol

At 24 h (n = 4 per group) or 7 days (n = 6 per group) after experiments, mice were re-anesthetized with 4% isoflurane and killed by ice-cold saline transcardial perfusion, followed by 10% buffered formalin phosphate perfusion and fixation of brains with subsequent embedding in paraffin at 2 weeks. Multiple 5-μm sections, 200 μm apart, from bregma −1.86 to −2.26, were prepared from each brain, and stained with hematoxylin and eosin (H&E; Thermo Scientific, Pittsburgh, PA). Additional 5-μm sections were obtained from the interval tissue and stained with Fluoro-Jade C (FJC; Chemicon, Temecula, CA) to evaluate for neuronal degeneration at 24 h (Schmued et al, 2005). Sections stained with FJC were assessed qualitatively. Hippocampal neuronal damage was quantified with 7-day cell counts in H&E sections by blinded evaluator using *Image J* (*http://rsb.info.nih.gov/ij/*). Cell counts were quantified in CA1 and CA3, and are reported as the average number of normal appearing neurons per 100-μm hippocampal pyramidal cell layer length. The 5-μm H&E sections taken from bregma −1.86 to −2.26 were also qualitatively evaluated by a neuropathologist (R.H.G.) blinded to treatment group.

Statistical analysis

Physiologic parameters and cell counts were compared between groups using one-way analysis of variance (ANOVA) and post-hoc tests with appropriate correction for multiple comparisons. All data are provided as mean ± standard error of the mean (SEM). The primary outcome parameter of the study was neuron counts in CA1 hippocamus ipsilateral to CCI (or in the left hippocampus in HS-only). Significance was determined by a p value of ≤ 0.05.

Results

Physiology

Table 1 provides a summary of important physiologic variables. MAP (the mean of all values for each group dur-

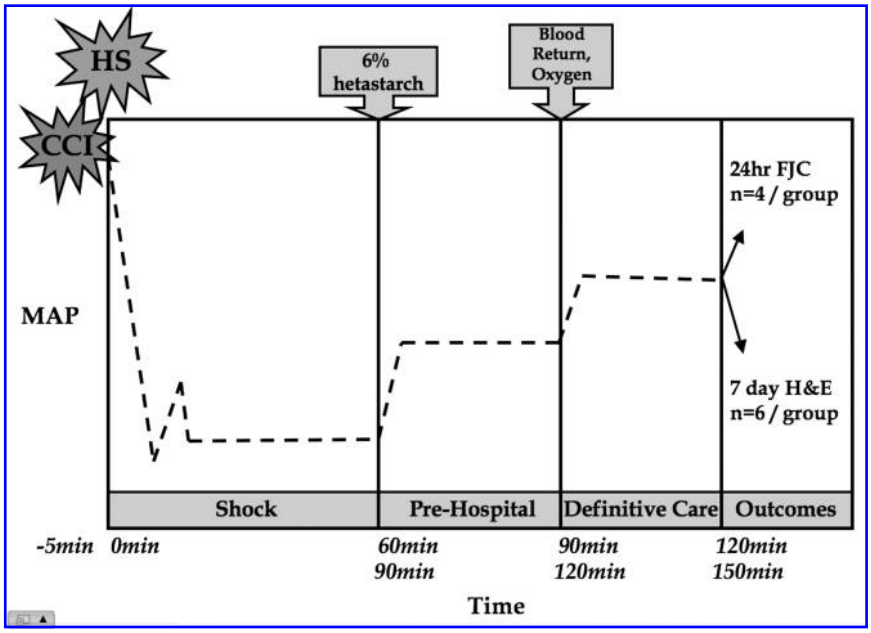

FIG. 1. Diagram depicting the overall scheme and timen course of experiment protocol used in this study (CCI, controlled cortical impact; HS, hemorrhagic shock; FJC, Fluoro-Jade C; H&E, hematoxylin and eosin; MAP, mean arterial blood pressure).

Table 1. Physiologic Data (Mean Arterial Blood Pressure, Hematocrit, Lactate, and Base Deficit)

	Baseline	*Shock*	*Definitive care*
MAP (mmHg)			
CCI only	85.4 ± 6.6	80.2 ± 4.0	77.0 ± 2.5
60 HS only	90.2 ± 3.6	35.5 ± 3.6	74.5 ± 5.4
90 HS only	89.8 ± 5.3	39.3 ± 4.1	72.8 ± 4.3
60 CCI + HS	88.5 ± 8.4	34.3 ± 5.0	69.9 ± 5.0
90 CCI + HS	85.4 ± 5.5	36.3 ± 6.4	69.9 ± 6.9
HCT (%)			
CCI only	38.7 ± 4.6	36.3 ± 4.5	32.4 ± 4.3
60 HS only	36.6 ± 1.4	27.6 ± 1.8	30.1 ± 1.9
90 HS only	35.7 ± 1.1	26.6 ± 1.7	29.3 ± 1.8
60 CCI + HS	37.1 ± 3.7	27.7 ± 3.7	28.5 ± 2.4
90 CCI + HS	36.8 ± 2.6	28.3 ± 2.0	28.3 ± 2.0
Lactate (mmol/L)			
CCI only	2.5 ± 0.1	2.4 ± 0.3	1.8 ± 0.2
60 HS only	2.2 ± 0.1	4.0 ± 0.4	1.6 ± 0.1
90 HS only	2.3 ± 0.1	3.2 ± 0.2	1.6 ± 0.1
60 CCI + HS	2.6 ± 0.2	3.5 ± 0.3	1.9 ± 0.2
90 CCI + HS	2.5 ± 0.2	4.2 ± 0.4	2.2 ± 0.1
Base deficit (mmol/L)			
CCI only	−4.7 ± 0.4	−5.2 ± 0.4	−5.7 ± 1.5
60 HS only	−4.7 ± 0.7	−6.9 ± 0.4	−4.8 ± 0.2
90 HS only	−4.7 ± 0.3	−6.6 ± 0.2	−5.4 ± 0.4
60 CCI + HS	−4.7 ± 0.3	−8.1 ± 0.6	−4.9 ± 0.5
90 CCI + HS	−4.7 ± 0.4	−7.2 ± 0.3	−5.1 ± 0.5

MAP, mean arterial blood pressure; HCT, hemocrit; CCI, controlled cortical impact; HS, hemorrhagic shock.

ing the HS interval) was significantly lower during *Shock* in all groups with HS compared to CCI only (60HS-only, 90HS-only, 60CCI+HS, and 90CCI+HS, respectively, all $p < 0.05$ versus CCI-only). In addition, the MAP during HS for all groups was within the target range of 30–40 mm Hg. During *Pre-Hospital* resuscitation, MAP increased into the target range of ≥50 mm Hg in all groups and was 50–60 mm Hg in all of the HS groups (with or without TBI, data not shown). In contrast, MAP in the CCI-only group was higher, as anticipated, at 77.3 ± 3.0 mm Hg. During *Definitive Care* resuscitation, MAP recovered to ≥70 mm Hg in the HS-only groups, while 60CCI+HS and 90CCI+HS were nearly 70 mm Hg at 69.9 ± 5.0 and 69.9 ± 6.9 mm Hg, respectively.

Hematocrit decreased by ~30% in mice subjected to HS and CCI+HS during *Shock* and *Pre-Hospital* phases; hematocrit partially recovered in all groups in *Definitive Care* (Table 1). Ostensibly, lack of complete recovery resulted from hemodilution from volume resuscitation, with hetastarch re-

Table 2. Physiologic Data (pH, $PaCO_2$, and PaO_2)

	Baseline	*Shock*	*Definitive care*
pH			
CCI only	7.34 ± 0.01	7.37 ± 0.01	7.33 ± 0.02
60 HS only	7.38 ± 0.01	7.40 ± 0.01	7.35 ± 0.01
90 HS only	7.36 ± 0.01	7.41 ± 0.01	7.33 ± 0.01
60 CCI + HS	7.36 ± 0.01	7.36 ± 0.02	7.34 ± 0.01
90 CCI + HS	7.37 ± 0.01	7.38 ± 0.01	7.31 ± 0.02
$paCO_2$ (torr)			
CCI only	37.0 ± 3.4	31.7 ± 3.9	35.2 ± 4.7
60 HS only	31.6 ± 2.4	25.3 ± 1.6	35.8 ± 2.2
90 HS only	33.6 ± 3.1	25.3 ± 1.5	36.7 ± 3.4
60 CCI + HS	33.5 ± 3.9	26.9 ± 1.8	36.4 ± 4.2
90 CCI + HS	33.3 ± 4.3	26.9 ± 1.9	40.0 ± 6.6
paO_2 (torr)			
CCI only	162.3 ± 7.9	79.4 ± 9.0	446.4 ± 25.0
60 HS only	163.0 ± 17.0	99.9 ± 8.5	441.1 ± 10.5
90 HS only	157.6 ± 13.7	93.2 ± 6.8	432.3 ± 18.4
60 CCI + HS	154.2 ± 14.3	87.6 ± 9.9	444.6 ± 27.2
90 CCI + HS	168.7 ± 12.3	92.2 ± 10.7	465.9 ± 19.9

CCI, controlled cortical impact; HS, hemorrhagic shock.

TABLE 3. PHYSIOLOGIC DATA (SODIUM, GLUCOSE, AND OXMOLALITY)

	Baseline	*Shock*	*Definitive care*
Sodium (mmol/L)			
CCI only	142.1 ± 0.3	142.6 ± 0.6	142.6 ± 0.7
60 HS only	142.6 ± 0.3	140.8 ± 0.2	143.5 ± 0.3
90 HS only	142.4 ± 0.4	140.2 ± 0.5	143.3 ± 0.2
60 CCI + HS	142.7 ± 0.4	140.4 ± 0.4	143.1 ± 0.5
90 CCI + HS	142.1 ± 0.5	140.5 ± 0.5	144.0 ± 0.5
Glucose (mg/dL)			
CCI only	88.8 ± 10.9	134.0 ± 19.9	87.1 ± 6.7
60 HS only	92.9 ± 7.2	169.0 ± 11.0	74.8 ± 8.1
90 HS only	101.9 ± 9.3	151.1 ± 12.2	71.6 ± 5.8
60 CCI + HS	94.7 ± 9.7	148.5 ± 20.6	73.8 ± 4.2
90 CCI + HS	87.6 ± 11.9	179.9 ± 10.2	85.4 ± 6.7
Osmolality (mOsm/kg)			
CCI only	290.0 ± 1.1	294.8 ± 1.7	292.3 ± 1.3
60 HS only	289.9 ± 1.2	293.2 ± 1.2	293.5 ± 0.9
90 HS only	293.2 ± 1.3	293.4 ± 1.8	296.3 ± 1.8
60 CCI + HS	291.2 ± 1.3	291.9 ± 1.7	293.0 ± 1.3
90 CCI + HS	291.9 ± 2.7	293.4 ± 1.8	296.3 ± 1.8

CCI, controlled cortical impact; HS, hemorrhagic shock.

quired to maintain MAP. Fluid requirements during *Pre-Hospital* and *Definitive Care* were 0.29 ± 0.1, 0.24 ± 0.1, 0.34 ± 0.1, and 0.34 ± 0.1 mL of 6% hetastarch in 60HS-only and 90HS-only versus 60CCI+HS and 90CCI+HS groups, respectively, and did not significantly differ between groups.

Not surprisingly, compared to CCI-only, groups subjected to HS or CCI+HS had higher lactate levels and greater base deficits during *Shock*; all measurements were taken at the same protocol time-point. These values were significant at $p = 0.05$ for CCI versus 60CCI+HS and 90CCI+HS groups for base deficit during *Shock*. For blood lactate levels, there was a predictable rise and fall during *Shock* and both resuscitation phases in 60HS, 90HS, and 60CCI+HS groups compared to CCI-only ($p = 0.05$); however, lactate levels in the 90CCI+HS group continued to be significantly albeit mildly higher during *Definitive Care* as well, likely reflecting continued lactate "wash-out" with resuscitation after prolonged HS.

All groups had similar trends in PaO_2 (Table 2); there was appropriate equilibration after room air administration and the expected increase with initiation of 100% oxygen during *Definitive Care*. $PaCO_2$ decreased in HS-only and CCI+HS groups during *Shock*, while pH did not, a difference likely

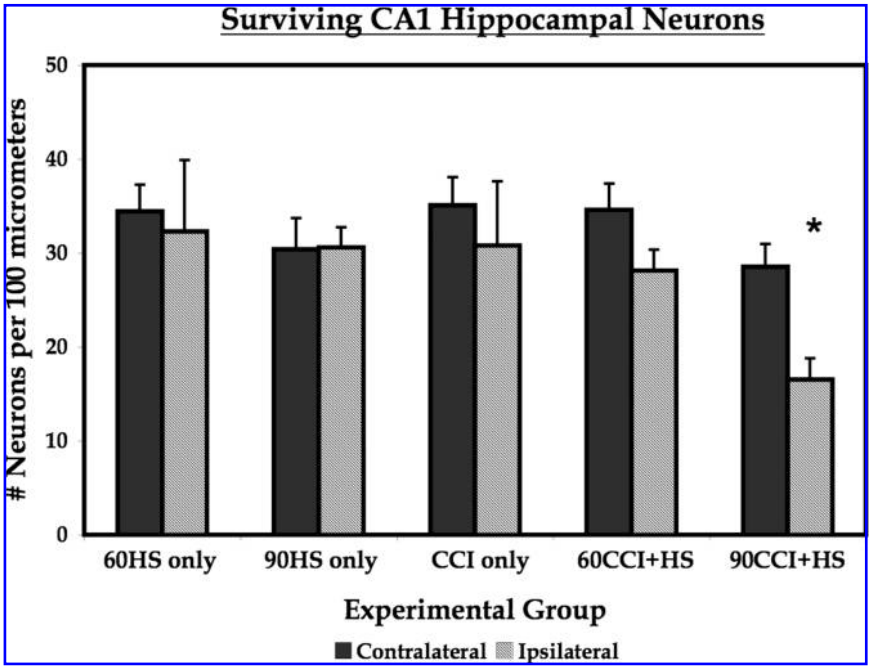

FIG. 2. Average number of surviving CA1 hippocampal neurons per 100-μm length for each experimental group, both ipsilateral (light bars) and contralateral (dark bars). Data are mean and SEM, $n = 6$ for each group. *$p < 0.05$ compared to all other groups (HS, hemorrhagic shock; CCI, controlled cortical impact).

related to compensatory hyperventilation. There were no significant differences between groups with regard to glucose, osmolality, sodium, potassium, ionized calcium, or ionized magnesium at any of the sampling times (Table 3, all data not shown).

Neuropathology

Hippocampal neuron counts. Surviving CA1 neuron counts in dorsal hippocampus ipsilateral to injury in the 90CCI+HS group were significantly reduced compared to all other study groups (Fig. 2). Average ipsilateral CA1 neuron counts 90CCI+HS were 16.5 ± 14.1 versus 30.8 ± 6.8, 32.3 ± 7.6, 30.6 ± 2.2, and 28.1 ± 2.2 neurons per 100-μm pyramidal cell layer length (CCI-only, 60HS-only, 90HS-only, and 60CCI+HS, respectively, all $p < 0.05$). There were no significant differences between groups for hippocampal neuron counts in CA3 ipsilateral to injury (Fig. 3) or in either CA1 or CA3 contralateral to injury, suggesting that CCI-only at this relatively mild level, HS-only, or combined injury did not produce significant neuronal loss in these hippocampal subfields. Examples of mice from the four insult groups are shown in Figure 4.

H&E neuropathologic survey. Review of 7-day H&E sections from 90CCI+HS mice consistently demonstrated hemorrhage and focal, full-thickness necrosis of the parietal cortex overlying the dorsal hippocampus ipsilateral to injury. Moderate acute eosinophilic degeneration was observed in the hippocampal neurons of the underlying dorsal subiculum, CA1, CA4, and dentate gyrus, particularly the dorsal blade. Occasional eosinophilic neurons were noted in CA3 of the hippocampus as well as in the dorsal thalamus. Scattered microglial and neutrophil infiltrates were present, as was neuropil vacuolation. The contralateral sides lacked abnormal histologic alterations in the 90CCI+HS group, as well as in all other study groups. Sections from the CCI-only and 60CCI+HS groups demonstrated identical full-thickness necrosis of the injured parietal cortex. However, unlike 90CCI+HS, eosinophilic neuron degeneration in CA1 and dentate gyrus was more mild in quality, and eosinophilic change in thalamic neurons was rare. HS-only sections revealed no evidence of neuronal damage. Additional selected images from the neuropathological survey are presented in Figure 5.

FluoroJade-C staining at 24 h post-insult. FJC positivity at 24 h was seen predominantly in CA1 and dentate gyrus and largely restricted to mice in the 90CCI+HS group (Fig. 5). This corresponded with regions of CA1 neuron loss at 7 days as assessed by H&E staining, corroborating neuronal degeneration in the observed areas of subsequent neuron loss. Rare FJC-positive neurons were seen in 60CCI+HS mice.

Discussion

We specifically chose a level of TBI that produced a cortical contusion that was just below the threshold for overt neuronal loss in the underlying dorsal hippocampus. We also selected a clinically relevant level of HS, based on the work of Carillo et al. (1998), with a MAP that we anticipated would not produce neurological injury in mice subjected to HS-only at the durations studied in our protocol. We were, however, surprised that 90 min rather that 60 min of HS was

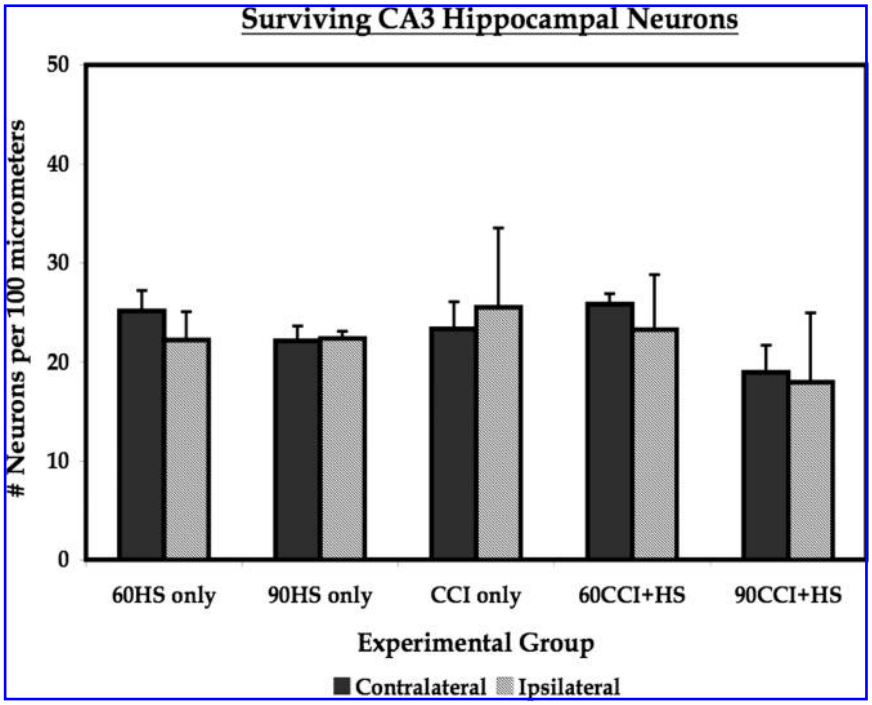

FIG. 3. Average number of surviving CA3 hippocampal neurons per 100-μm length for each experimental group, both ipsilateral (light bars) and contralateral (dark bars). Data are mean and SEM, $n = 6$ for each group. There were no differences between groups (HS, hemorrhagic shock; CCI, controlled cortical impact).

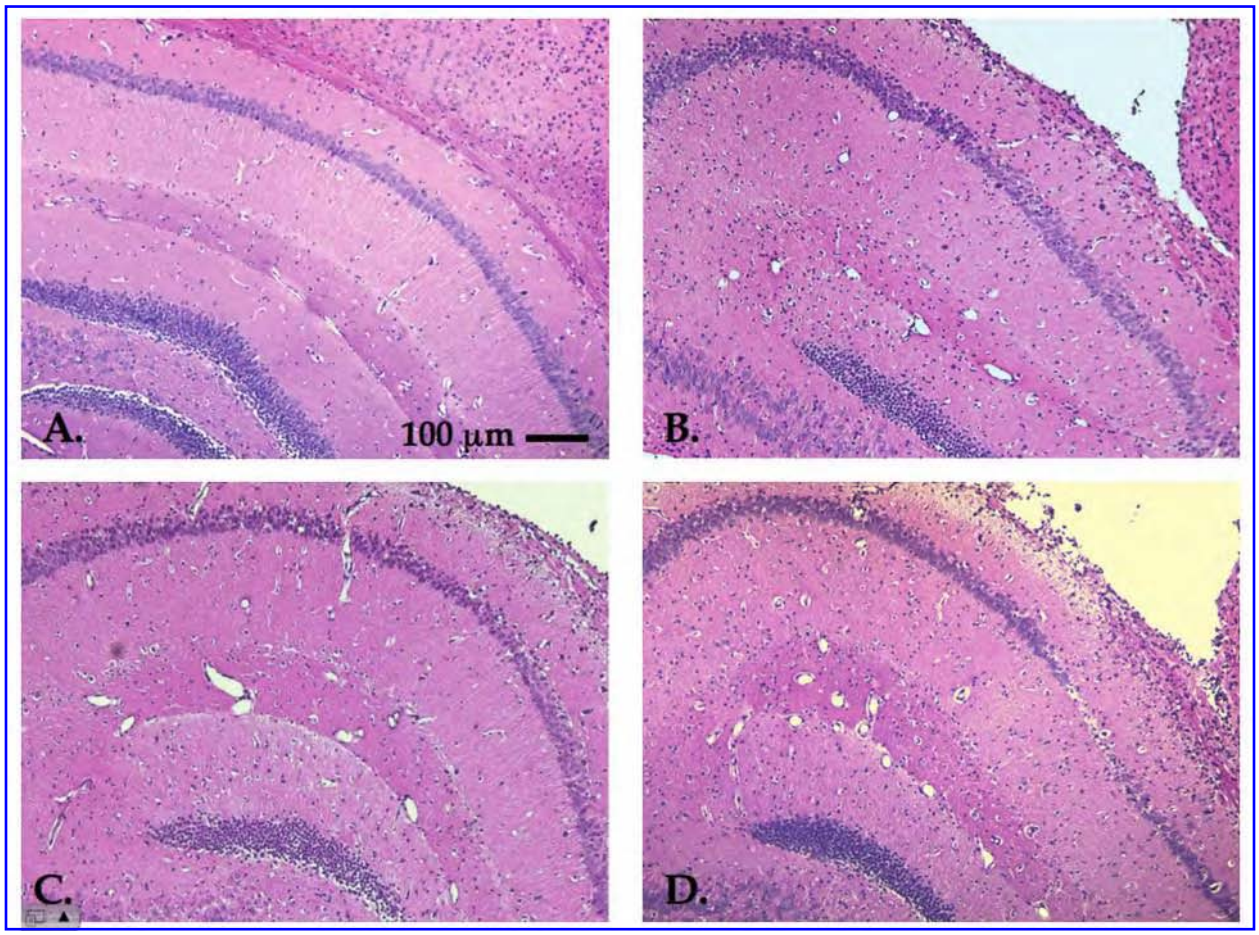

FIG. 4. Representative microphotographs (original magnification, ×20), stained with hematoxylin and eosin (H&E), depicting the CA1 hippocampal subfield in 90HS-only (**A**), CCI-only (**B**), 60CCI+HS (**C**), and 90CCI+HS (**D**). 60HS is not shown. Pyramidal neuron loss is evident within the medial region of CA1 in the 90CCI+HS group. HS, hemorrhagic shock; CCI, controlled cortical impact.

required to exacerbate neuronal death after the chosen level of CCI. When *Shock* duration was extended to 90 min, we observed a neuronal loss pattern previously well-defined in experiments of ischemia and hypoxemia, namely, ~60% loss of selectively vulnerable CA1 pyramidal neurons in the dorsal hippocampus by 7 days after the insult. Whether or not additional neuronal loss would be seen at longer outcomes remains to be determined. The duration of HS required to produce hippocampal neuronal death after TBI was longer than anticipated, since in studies of CCI in rats, addition of 30 min of hypoxemia (PaO_2 ~ 40 mm Hg) was sufficient (Clark et al., 1997). However, systemic hypoxemia in those studies resulted in the development of hypotension after ~15–20 min, and combined hypoxemia and hypotension is likely to be particularly deleterious (Siesjo, 1978). The fact that hypotension often develops in TBI models where secondary hypoxemia is superimposed is, in our opinion, underappreciated. In addition, those studies with hypoxemia in rats used a relatively greater injury severity level than used in our study, which could also importantly increase the level of vulnerability of the injured hippocampus to a secondary insult. Given that the normal MAP in mice anesthetized with isoflurane in our model was ~85–90 mm Hg, our studies indicate that HS to a MAP that is 50–60% below baseline can be tolerated for 60 min after TBI, suggesting that there may be a greater than anticipated therapeutic time window for successful resuscitation to mitigate deleterious consequences of a secondary insult in the traumatically injured brain. This finding is similar to the work of Stern et al. (2000), who reported that acute cerebral hemodynamic parameters were preserved in pigs after FPI, despite HS to a MAP of 30 mm Hg for a period of 60 min. Longer periods of shock were not studied in that model.

In pilot studies, to produce an identical level of hypotension in mice subjected to either HS or CCI+HS, it was necessary to use a greater degree of hemorrhage in HS-only mice (2.7 mL/100 g blood withdrawal versus 2.0 mL/100 g in HS-only and CCI+HS groups, respectively). This relationship between TBI and reduced tolerance to HS has been reported (Law et al., 1996; Yuan and Wade, 1992) and suggests a systemic consequence of CNS trauma on blood pressure regulation. Chesnut et al. (1998) observed hypotension in humans with isolated TBI and without significant extracerebral injury. Mahoney et al. (2003) confirmed and expounded on this observation, citing possible brainstem involvement, altered autonomic tone, or massive catecholamine surge with ensu-

ing transmitter depletion, receptor saturation, and consequent myocardial depression and cardiovascular collapse. Using a rat model of FPI and HS, Law et al. (1996) showed that rodents subjected to either isolated brain injury or HS were able to adjust vascular tone to maintain MAP; however, when these insults were combined, compensatory vasoconstriction during shock failed to occur. Yuan et al. (1992) showed both suppression of spontaneous MAP recovery in rats subjected to combined injury and attenuation of the MAP response to fluid resuscitation. We initially attempted to use higher MAP resuscitation goals in pilot studies, ≥60 mm Hg MABP in the *Pre-Hospital* phase and ≥80 mm Hg (normotension) MAP in the *Definitive Care* phase. However, targeting these goals resulted in uniform mortality in unintubated, spontaneously breathing mice. We observed frank pulmonary edema, possibly neurogenic in origin or due to myocardial depression. The possibility of cerebral edema in the face of aggressive fluid resuscitation also cannot be excluded, as we have not yet measured ICP in this mouse model. In this initial study, we were concerned that addition of invasive ICP monitoring would potentially compromise long-term survival in these small rodents. Future studies of this important parameter are needed. Nevertheless, our data clearly confirm that the cardiovascular system is sensitized to the hemodynamic consequences of HS by a preceding TBI.

We chose to use hetastarch as our resuscitation fluid in this model, since it is the current standard of care in the U.S. Army for combat casualty resuscitation (Holcomb, 2003). Other resuscitation fluids will need to be tested in this model, since they may exhibit differing efficacies in neuroprotection even if the same MAP targets are used.

While we did not evaluate CBF in these mice, the literature is replete with evidence of cerebrovascular dysregulation and regional blood flow reductions after TBI. Posttraumatic hypoperfusion after CCI in rats has been reported across laboratories and assessment techniques (Bryan et al., 1995; Hendrich et al., 1999), and loss of blood pressure autoregulation of CBF after TBI has been reported (Lewelt et al., 1980), potentially allowing exacerbation of cerebral hypoperfusion with even modest reductions in MAP. In injured regions with increased metabolic requirements, small reductions in CBF, or perhaps simply the failure to increase CBF and substrate delivery to match demands could be sufficient to damage vulnerable neurons when thresholds of energy failure are reached. Currently, we are using perfusion magnetic resonance imaging to study CBF in this model (Dennis et al., 2006).

In the CCI model in rodents, marked cerebral hyperglycolysis has been reported in the hippocampus underlying the contusion (Hovda et al., 1995; Statler et al., 2003). In HS, anemia accompanies the reduction in MAP, and we observed a significant reduction in HCT; therefore, the anticipated reduction in oxygen and substrate delivery produced by hypoperfusion would be amplified. The contribution of anemia to the exacerbation of neuronal injury in combined TBI and HS, however, remains to be defined.

We used isoflurane anesthesia in our model. Isoflurane by inhalation provides a consistent level of anesthesia that can be readily titrated and promptly discontinued. However, isoflurane reduces cerebral metabolic demands, provides some degree of CBF promotion (particularly in subcortical structures), and is neuroprotective after TBI (Statler et al., 2000, 2006). Severe TBI in humans is generally not treated with sedatives or anesthetics in the field. Nevertheless, it is necessary to provide anesthesia in animal models of TBI, and thus our model may underestimate the amount of damage that a similar level of TBI and HS would produce in the clinical setting. Studies of combined TBI plus HS in this model using less protective and more clinically relevant anesthetics and/or analgesics such as fentanyl are warranted.

Most studies of combined experimental TBI and HS have been carried out in large animals and have been focused on the influence of resuscitation fluids on intracranial dynamics, including ICP and CBF (Gibson et al., 2002; Shackford, 1997; Bedell et al., 1998). Long-term outcomes in these models are extremely expensive and have generally not been investigated; thus, the impact of HS on delayed neuronal death has been subjected to limited investigation. There have been a few studies in rodent models, however, that are noteworthy.

In rats, using combined mild-moderate FPI and HS to a MAP of 50–60 mm Hg for 30 min, Schütz et al. (2006) did not observe either an increase in cerebral edema or an exacerbation of cortical tissue loss when FPI+HS was compared to FPI alone. They did, however, observe a delay in cognitive recovery in FPI+HS rats, suggesting subtle cerebral injury not necessarily related to edema or cortical tissue damage alone. They used a milder level of hypotension compared to our model, as well as a shorter shock duration, and without re-infusion of shed blood in the resuscitation phase, clinical extrapolation may be limited. Failure to treat the marked anemia that accompanies HS after TBI would be deemed to be outside of the current standard of care in TBI management. Matsushita et al. (2001) performed a similar protocol in rats also using moderate FPI and HS to a MAP of 60 mm Hg for 30 min. However, they observed exacerbated contusion size in the posterior parietal cortex in FPI+HS rats when compared to FPI alone; they did not report assessment of the hippocampus. Similarly, with a different experimental TBI mechanism, impact acceleration, Ito et al. (1996) added 30 min of hypoxemia and hypotension (a PaO_2 of 40 mm Hg and MAP of 30 mm Hg, respectively) and used diffusion-weighted imaging to discern apparent diffusion coefficients (ADCs) and extrapolate cytotoxic versus extracellular edema. Combined injury rats demonstrated marked and sustained increases in ICP and reduced CBF as well as reduced ADC, consistent with cytotoxic edema, despite resuscitation, when compared to controls. When Barzó et al. (1997) used this identical protocol but added an additional combined injury group of impact acceleration, hypoxemia to a PaO_2 of 40 mm Hg and hypotension with a MAP of 40–50 mm Hg, they observed recovery of both ADC and clinical condition in the MAP 40–50 mm Hg combined insult group, while the MAP 30–40 mm Hg group did not recover, and progressed to death. This suggested a critical MAP threshold of 30–40 mm Hg, which was the MAP seen in our study during HS in all groups exposed to shock. Similar to the work of Clark et al., using CCI and secondary hypoxic insult in rats, the earlier work of Ishige et al. (1987c) in FPI used 2,3,5-triphenyltetrazolium chloride to reveal an ischemic area surrounding the contusion not seen with either isolated FPI or hypoxemia alone. They also used MRI to confirm extension of the contusion and surrounding edema in combined insult rats versus TBI alone and observed a decrease in CBF in the entire ipsilateral cortex in these rats. Further work by Ishige

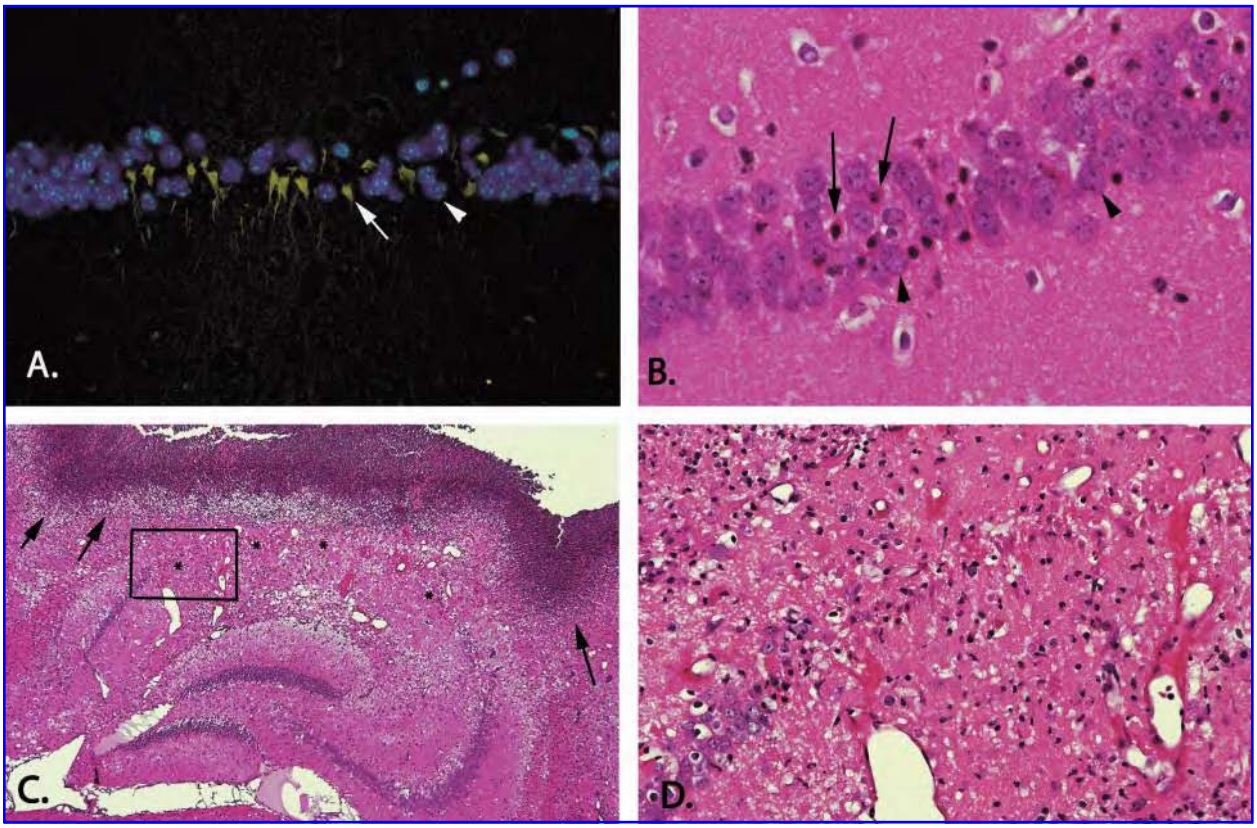

FIG. 5. (**A**) Representative medium-high-power micrograph (×20 objective) of a 90CCI+HS mouse hippocampus demonstrating normal pyramidal neurons (arrowhead) stained blue with DAPI and degenerating neurons stained yellow-green with Fluoro-Jade C (FJC; long arrow) within the CA1 subfield at 24 h. (**B**) High-power micrograph (×40 objective) of a hematoxylin and eosin (H&E)–stained section demonstrating normal pyramidal neurons (arrowheads) interspersed with degenerating eosinophilic neurons (long arrows) at 7 days in the CA1 hippocampal subfield of a 90CCI+HS mouse. (**C**) Low-power microphotograph (×4 objective) of the most severe spectrum of hippocampal damage sustained by several mice in the 90CCI+HS group. Robust microglial and PMN infiltration is noted along the superior aspect (arrows), and there is substantial loss of neurons in the CA1 subfield (asterisks). (**D**) Higher magnification (×20 objective) of the boxed region in C demonstrates the absence of viable pyramidal neurons and loss of architecture within much of the CA1 subfield in this example. CCI, controlled cortical impact; HS, hemorrhagic shock; PMN, polymorphonuclear leukocyte.

et al. (1988) used phosphocreatine (PCr)/inorganic phosphate ratios obtained by in vivo phosphorus-31 magnetic resonance spectroscopy as evidence for changes in high-energy metabolite concentrations and observed that depletion of high-energy metabolites was markedly accelerated by combined FPI and hypoxemia in a dose-dependent fashion, versus FPI alone. They added a hypotensive insult (MAP 30–40 mmHg) to the combination of FPI and hypoxemia, and observed even further depletion of high-energy phosphates in the brain, again consistent with the MAP used in our study. These studies support the possibility that HS exacerbated energy failure in the pericontusional brain regions, including hippocampus, thus triggering neuronal death in our model.

We re-infused autologous shed blood, which would not be available for clinical use. It is recognized that massive transfusion of packed red blood cells can produce a number of unwanted side effects (i.e., immunosuppression, hyperkalemia, coagulopathy) and that complications from transfusion are generally related to the duration of blood storage prior to use. Thus, one might anticipate that secondary injury and complications are underestimated in our mouse model relative to the human condition. Recently, in the setting of massive blood loss in combat casualty care, greater emphasis has been placed on the use of fresh whole blood.

Finally, we focused our histopathological examination to hippocampal neuronal counts given the anticipated vulnerability of that brain region to secondary ischemic insults. However, further studies are needed to assess cortical damage and other brain regions, given the fact that our qualitative survey suggested the possibility of enhanced damage in other structures.

While technically challenging in mice, an experimental model of combined TBI plus HS is feasible with reasonable clinical fidelity. This initial study characterizes a new model which, given the ready availability of genetic variant mice, is unique in its potential for application to mechanistic and therapeutic study of this injury combination.

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Author Disclosure Statement

No conflicting financial interests exist.

References

Barron, K.D., Dentinger, M.P., Kimelberg, H.K., Nelson, L.R., Bourke, R.S., Keegan, S., Mankes, R., and Cragoe, E.J., Jr. (1988). Ultrastructural features of a brain injury model in cat. I. Vascular and neuroglial changes and the prevention of astroglial swelling by fluorenyl (aryloxy) alkanoic acid derivative (L-664, 711). Acta Neuropapthol. 75, 295–307.

Barzó, P., Marmarou, A., Fatouros, P., Corwin, F., and Dunbar, J.G. (1997). Acute blood-brain barrier changes in experimental closed head injury as measured by MRI and Gd-DTPA. Acta Neurochir. Suppl. 70, 243–246.

Bedell, E.A., DeWitt, D.S., and Prough, D.S. (1998). Fentanyl infusion preserves cerebral blood flow during decreased arterial blood pressure after traumatic brain injury in cats. J. Neurotrauma 15, 985–992.

Bryan, R.M., Cherian, L., and Robertson, C. (1995). Regional cerebral blood flow after controlled cortical impact injury in rats. Anesth. Analg. 80, 687–695.

Carillo, P., Takasu, A., and Safar, P. (1998). Severe hemorrhagic shock and resuscitation in rats does not cause subtle brain damage. J. Trauma 45, 239–249.

Chesnut, R.M., Marshall, L.F., Klauber, M.R., Blunt, B.A., Baldwin, N., Eisenberg, H.M., Jane, J.A., Marmarou, A., and Foulkes, M.A. (1993). The role of secondary brain injury in determining outcome from severe head injury. J. Trauma 34, 216–222.

Chesnut, R.M., Gautille, T., Blunt, B.A., Klauber, M.R., and Marshall, L.F. (1998). Neurogenic hypotension in patients with severe head injuries. J. Trauma 44, 958–963.

Clark, R.S.B., Kochanek, P.M., Dixon, C.E., Chen, M., Marion, D.W., Heineman, S., DeKosky, S.T., and Graham, S.H. (1997). Early neuropathologic effects of mild or moderate hypoxemia after controlled cortical impact injury in rats. J. Neurotrauma 14, 179–189.

Dennis, A.M., Foley, L.M., Melick, J.A., Hitchens, T.K., Jenkins, L.W., Clark, R.S., Ho, C., and Kochanek, P.M. (2006). Effect of hemorrhagic shock on cerebral blood flow in experimental traumatic brain injury: magnetic resonance imaging assessment. Crit. Care Med. 34, A5.

DeWitt, D.S., Jenkins, L.W., and Prough, D.S. (1995). Enhanced vulnerability to secondary ischemic insults after experimental traumatic brain injury. New Horiz. 3, 376–383.

DeWitt, D.S., Prough, D.S., Taylor, C.L., and Whitley, J.M. (1992a). Reduced cerebral blood flow, oxygen delivery, and electroencephalographic activity after traumatic brain injury and mild hemorrhage in cats. J. Neurosurg. 76, 812–821.

DeWitt, D.S., Prough, D.S., Taylor, C.L., Whitley, J.M., Deal, D.D., and Vines, S.M. (1992b). Regional cerebrovascular responses to progressive hypotension after traumatic brain injury in cats. Am. J. Physiol. 263, 1276–1284.

Foley, L.M., Hitchens, T.K., Melick, J., Bayır, H., Ho, C., and Kochanek, P.M. (2008). Effect of inducible nitric oxide synthase on cerebral blood flow after experimental traumatic brain injury in mice. J. Neurotrauma 25, 299–310.

Gawande, A. (2004). Casualties of war- military care for the wounded from Iraq and Afghanistan. N. Engl. J. Med. 351, 2471–2475.

Gibson, J.B., Maxwell, R.A., Schweitzer, J.B., Fabian, T.C., and Proctor, K.G. (2002). Resuscitation from severe hemorrhagic shock after traumatic brain injury using saline, shed blood, or a blood substitute. Shock 17, 234–244.

Glass, T.F., Fabian, M.J., Schweitzer, J.B., Weinberg, J.A., and Proctor, K.G. (1999). Secondary neurologic injury resulting from nonhypotensive hemorrhage combined with mild traumatic brain injury. J. Neurotrauma 16, 771–782.

Gopinath, S.P., Robertson,C.S., Contant, C.F., Hayes, C., Feldman, Z., Narayan, R.K., and Grossman, R.G. (1994). Jugular venous desaturation and outcome after head injury. J. Neurol. Neurosurg. Psychiatry 57, 717–723.

Gutierrez de Ceballos, J.P., Turégano Fuentes, F., Perez Diaz, D., Sanz Sanchez, M., Martin Llorente, C., and Guerrero Sanz, J.E. (2005). Casualties treated at the closest hospital in the Madrid, March 11, terrorist bombings. Crit. Care Med. 33, Suppl 1, S107–S112.

Hendrich, K.S., Kochanek, P.M., Williams, D.S., Schiding, J.K., Marion, D.W., and Ho, C. (1999). Early perfusion after controlled cortical impact in rats: quantification by arterial spin-labeling and the influence of spin-lattice relaxation time heterogeneity. Magn. Reson. Med. 42, 673–681.

Holcomb, J.B. (2003). Fluid resuscitation in modern combat casualty care: lessons learned from Somalia. J. Trauma 54, S46–S51.

Hovda, D.A., Lee S.M., Smith M.L., Von Stuck, S., Bergsneider, M., Kelly, D., Shalmon, E., Martin, N., Caron, M., Mazziotta, J., et al. (1995). The neurochemical and metabolic cascade following brain injury: moving from animal models to man. J. Neurotrauma 12, 903–906.

Ishige, N., Pitts, L.H., Hashimoto, T., Nishimura, M.C., and Bartkowski, H.M. (1987a). Effect of Hypoxia on traumatic brain injury in rats: Part 1. Changes in neurological function, electroencephalograms, and histopathology. Neurosurgery 20, 848–853.

Ishige, N., Pitts L.H., Pogliani, L., Hashimoto, T., Nishimura, M.C., Bartkowski, H.M., and James, T.L. (1987b). Effect of hypoxia on traumatic brain injury in rats: Part 2. Changes in high energy phosphate metabolism. Neurosurgery 20, 854–858.

Ishige, N., Pitts, L.H., Berry, I., Carlson, S.G., Nishimura, M.C., Moseley, M.E., and Weinstein, P.R. (1987c). The effect of hypoxia on traumatic brain injury in rats: alterations in neurologic function, brain edema, and cerebral blood flow. J. Cereb. Blood Flow Metab. 7, 759–767.

Ishige, N., Pitts, L.H., Berry, I., Nishimura, M.C., and James, T.L. (1988). The effect of hypovolemic hypotension on high-energy phosphate metabolism of traumatized brain in rats. J. Neurosurg. 68, 129–136.

Ito, J., Marmarou, A., Barzó, P., Fatouros, P., and Corwin, F. (1996). Characterization of edema by diffusion-weighted imaging in experimental traumatic brain injury. J. Neurosurg. 84, 97–103.

Jenkins, L.W., Marmarou, A., Lewelt, W., and Becker, D.P. (1986). Increased vulnerability of the traumatized brain to early ischemia, in: *Mechanisms of Secondary Brain Damage*. A. Baethmann, K.G. Go, A. Unterberg (eds), Plenum Press: New York, pps. 273–281.

Jenkins, L.W., Moszynski, K., Lyeth, B.G., Lewelt, W., DeWitt, D.S., Allen, A., Dixon, C.E., Povlishock, J.T., Majewski, T.J., Clifton, G.L., et al. (1989). Increased vulnerability of the mildly traumatized rat brain to cerebral ischemia: the use of con-

trolled secondary ischemia as a research tool to identify common or different mechanisms contributing to mechanical and ischemic brain injury. Brain Res. 477, 211–224.

Kirino, T. (1982). Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res. 239, 57–69.

Kochanek, P.M., Vagni, V.A., Janesko, K.L., Washington, C., Crumrine, P., Garman, R.H., Jenkins, L.W., Clark, R.S.B., Homanics, G., Dixon, C.E., Schnermann, J., and Jackson, E.K. (2006). Adenosine A1 receptor knockout mice develop lethal status epilepticus after experimental traumatic brain injury. J. Cereb. Blood Flow Metab. 26, 565–575.

Kotapka, M.J., Graham, D.I., Adams, J.H., and Genarelli, T.A. (1994). Hippocampal pathology in fatal human head injury without high intracranial pressure. J. Neurotrauma 11, 317–324.

Law, M.M., Hovda, D.A., and Cryer, H.G. (1996). Fluid percussion brain injury adversely affects control of vascular tone during hemorrhagic shock. Shock 6, 213–217.

Lewelt, W., Jenkins, L.W., and Miller, J.D. (1980). Autoregulation of cerebral blood flow after experimental fluid percussion injury of the brain. J. Neurosurg. 53, 500–511.

Lowenstein, D.H., Thomas, M.J., Smith, D.H., and McIntosh, T.K. (1992). Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. J. Neuroscience 12, 4846–4853.

Matsushita, Y., Bramlett, H.M., Kuluz, J.W., Alonso, O., and Dietrich, W.D. (2001). Delayed hemorrhagic hypotension exacerbates the hemodynamic and histopathologic consequences of traumatic brain injury in rats. J. Cereb. Blood Flow Metab. 21, 847–856.

Mahoney, E.J., Biffl, W.L., Harrington, D.T., and Cioffi, W.G. (2003). Isolated brain injury as a cause of hypotension in the blunt trauma patient. J. Trauma 55, 1065–1069.

Myers, S.I., Hernandez, R., and Miller, T.A. (1994). Differing effects of anesthetics on splanchnic arterial blood flow during hemorrhagic shock. J. Appl. Physiol. 76, 2304–2309.

Nelson, L.R., Bourke, R.S., Popp, A.J., Cragoe, E.J., Signorelli, A., Foster, V.V., and Creel, W. (1979). Evaluation of treatment modalities in severe head injuries using an animal model, in: *Neural Trauma*. A.J. Popp, R.S. Bourke, L.R. Nelson, H.K. Kimelberg (eds), Raven Press: New York, pps. 297–311.

Nelson, T.J., Wall, D.B., Stedje-Larsen, E.T., Clark, R.T., Chambers, L.W., and Bohman, H.R. (2006). Predictors of mortality in close proximity blast injuries during Operation Iraqi Freedom. J. Am. Coll. Surg. 202, 418–422.

Rosner, M.J., Newsome, H.H., and Becker, D.P. (1984). Mechanical brain injury: the sympathoadrenal response. J. Neurosurg. 61, 76–86.

Schmued, L.C., Stowers, C.C., Scallet, A.C., and Xu, L. (2005). Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. Brain Res. 1035, 24–31.

Schütz, C., Stover, J.F., Thompson, H.J., Hoover, R.C., Morales, D.M., Schouten, J.W., McMillan, A., Soltesz, K., Motta, M., Spangler, Z., Neugebauer, E., and McIntosh, T.K. (2006). Acute, transient hemorrhagic hypotension does not aggravate structural damage or neurologic motor deficits but delays the long-term cognitive recovery following mild to moderate traumatic brain injury. Crit. Care Med. 34, 492–501.

Shackford, S.R. (1997). Effect of small-volume resuscitation on intracranial pressure and related cerebral variables. J. Trauma 42, Suppl. 5, S48–S53.

Siesjo, B.K. (1978). Hypoxia, in: *Brain Energy Metabolism*. John Wiley & Sons: Chichester, UK, pps. 398–452.

Sinz, E.H., Kochanek, P.M., Dixon, C.E., Clark, R.S.B., Carcillo, J.A., Watkins, S.C., Schiding, J., Carlos, T.M., and Billiar, T.R. (1999). Inducible nitric oxide synthase is an endogenous neuroprotectant after traumatic brain injury in rats and mice. J. Clin. Invest. 104, 647–656.

Statler, K.D., Kochanek, P.M., Dixon, C.E., Alexander, H.L., Warner, D.S., Clark, R.S., Wisniewski, S.R., Graham, S.H., Jenkins, L.W., Marion, D.W., and Safar, P.J. (2000). Isoflurane improves long-term neurologic outcome versus fentanyl after traumatic brain injury in rats. J. Neurotrauma 17, 1179–1189.

Statler, K.D., Janesko, K.L., Melick, J.A., Clark, R.S.B., Jenkins, L.W., and Kochanek, P.M. (2003). Hyperglycolysis is exacerbated after traumatic brain injury with fentanyl versus isoflurane anesthesia in rats. Brain Res. 994, 37–43.

Statler, K.D., Alexander, H., Vagni, V.A., Dixon, C.E., Clark, R.S., Jenkins, L., and Kochanek, P.M. (2006). Comparison of seven anesthetic agents on outcome after experimental traumatic brain injury in adult, male rats. J. Neurotrauma 23, 97–108.

Stern, S.A., Zink, B.J., Mertz, M., Wang, X., and Dronen, S.C. (2000). Effect of initially limited resuscitation in a combined model of fluid-percussion brain injury and severe uncontrolled hemorrhagic shock. J. Neurosurgery 93, 305–314.

Vatner, S.F. (1974). Effects of hemorrhage on regional blood flow distribution in dogs and primates. J. Clin. Invest. 54, 225–235.

Whalen, M.J., Carlos, T.M., Dixon, C.E., Wisniewski, S.R., Schiding, J.K., Clark, R.S.B., Baum, E., Marion, D.W., and Kochanek, P.M. (1999. Effect of traumatic brain injury in mice deficient in intercellular adhesion molecule-1: assessment of histopathologic and functional outcome. J. Neurotrauma 16, 299–309.

Yuan, X.Q., and Wade, C.E. (1992). Traumatic brain injury attenuates the effectiveness of lactated ringers solution resuscitation of hemorrhagic shock in rats. Surg. Gyn. Obst. 174, 305–312.

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Short Communication

Resuscitation of Traumatic Brain Injury and Hemorrhagic Shock with Polynitroxylated Albumin, Hextend, Hypertonic Saline, and Lactated Ringer's: Effects on Acute Hemodynamics, Survival, and Neuronal Death in Mice

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Abstract

Outcome after traumatic brain injury (TBI) is worsened by hemorrhagic shock (HS), but the optimal resuscitation approach is unclear. In particular, treatment of TBI patients with colloids remains controversial. We hypothesized that resuscitation with the colloids polynitroxylated albumin (PNA) or Hextend (HEX) is equal or superior to resuscitation with the crystalloids hypertonic (3%) saline (HTS) or lactated Ringer's solution (LR) after TBI plus HS in mice. C57/BL6 mice (n = 30) underwent controlled cortical impact (CCI) and 90 min of volume-controlled HS (2 mL/100 g). The mice were randomized to resuscitation with LR, HEX, HTS, or PNA, followed by 30 min of test fluid administration targeting a mean arterial pressure (MAP) of >50 mm Hg. Shed blood was re-infused to target a MAP >70 mm Hg. At 7 days post-insult, hippocampal neuron counts were assessed in hematoxylin and eosin–stained sections to quantify neuronal damage. Prehospital MAP was higher, and prehospital and total fluid requirements were lower in the PNA and HEX groups ($p < 0.05$ versus HTS or LR). Also, 7-day survival was highest in the PNA group, but was not significantly different than the other groups. Ipsilateral hippocampal CA1 and CA3 neuron loss did not differ between groups. We conclude that the colloids PNA and HEX exhibited more favorable effects on acute resuscitation parameters than HTS or LR, and did not increase hippocampal neuronal death in this model.

Key words: colloid; head injury; nitroxide; oxidative stress; secondary insult

Introduction

Secondary insults after traumatic brain injury (TBI) increase morbidity and mortality, and the combination of TBI plus hemorrhagic shock (HS) is particularly deleterious. Miller and Becker (1982) first reported that hypotension (systolic blood pressure <90 mm Hg) worsened outcome after TBI. Chesnut and associates (1993) reported a correlation between hypotension and hypoxemia and increased morbidity/mortality after TBI in humans, with hypotension being the most critical parameter. These observations have been confirmed in experimental studies, in which secondary insults also worsened brain injury. Controlled cortical impact (CCI) with superimposed ischemia reduced cerebral blood flow (CBF) (Giri et al., 2000) and increased hippocampal neuronal loss (Cherian et al., 1996) versus ischemia alone. Jenkins and colleagues (1989) noted increased CA1 neuronal death by combining hemorrhagic hypotension with TBI in rats, and Matsushita and co-workers (2001) reported an increase in contusion area by hemorrhagic shock after fluid percussion injury in rats. Thus clinical and experimental evidence supports an association between secondary insults and increased morbidity and mortality after TBI.

The optimal fluid for resuscitation of TBI plus HS remains unclear. Characteristics of the ideal resuscitation fluid include ease of transport and administration in the pre-hospital setting, small infusion volumes to minimize cerebral edema, prevention of acute causes of mortality, and attenuation of

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secondary injury. Traditional acute resuscitation solutions for TBI plus HS include lactated Ringer's solution (LR) or Hextend® (HEX; Hospira, Lake Forest, IL). More novel resuscitation solutions under evaluation include hypertonic (3%) saline (HTS) and polynitroxylated albumin (PNA; SynZyme Technologies, Irvine, CA), among others. Isotonic crystalloids, particularly LR, are used for resuscitation in civilian trauma, but often require large volumes to maintain the desired blood pressure. HEX is the preferred fluid for resuscitation in combat casualty care. In several animal models, resuscitation with HEX required less volume and improved cerebrovascular function versus resuscitation with crystalloid (Crookes et al., 2004; Kelly et al., 2003; King et al., 2004). With regard to more novel solutions, there is extensive pre-clinical and clinical experience evaluating the use of HTS for resuscitation. Prough and associates (1991) and others have shown that use of HTS in experimental hemorrhagic hypotension restores hemodynamics and improves microcirculation. In animal models of TBI, HTS also improved CBF and lowered intracranial pressure (ICP) versus LR (Walsh et al., 1991; Shackford et al., 1992). Finally, PNA is a novel compound composed of 55 nitroxide moieties covalently linked to albumin that is administered as a 10% solution. Its stable nitroxyl radicals mimic superoxide dismutase (SOD) and catalase, and detoxify reactive oxygen species (Li et al,. 2002). PNA improved survival in a rat HS model and reduced lesion size in experimental stroke (Kentner et al., 2002; Beaulieu et al., 1998). It has not, however, been evaluated in combined TBI plus HS.

Recently, controversy about the optimal resuscitation fluid in TBI was raised by the SAFE study (Myburgh et al., 2007), which included a post-hoc analysis of the use of albumin versus saline in TBI victims. It suggested that the use of albumin in patients with TBI increased mortality versus saline, but no mechanism for the increased mortality seen with colloid use was presented. A recent report by Baker and colleagues (2008) in experimental TBI in rats challenged this finding, and showed enhanced electrophysiological recovery with albumin versus saline resuscitation, although the neuropathology was not assessed.

We recently developed a clinically relevant mouse model of TBI plus HS that allows us to evaluate acute hemodynamics, 7-day survival, and long-term neuropathology (Dennis et al., 2009). At the injury level used, CA1 neuronal death was seen only in combined CCI plus HS, but not in CCI or HS alone. We now use this model to evaluate the resuscitation of TBI plus HS using several traditional and novel fluids. We hypothesized that resuscitation with the colloids PNA or HEX would require smaller volumes than the crystalloids HTS or LR to reach resuscitation goals and produce higher mean arterial pressures (MAPs) in the resuscitation phase. We also hypothesized that the colloids PNA or HEX versus the crystalloids HTS or LR would not worsen 7-day survival or hippocampal neuronal death.

Methods

The Institutional Animal Care and Use Committee of the University of Pittsburgh School of Medicine approved this study. Male C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME), 12–15 weeks of age and weighing 22–29 grams, were housed under controlled environmental conditions and allowed *ad libitum* food and water until the study began.

Anesthesia was induced via nose cone with 4% isoflurane in oxygen, and maintained with 1% isoflurane in a 2:1 N_2O/oxygen mixture. Under sterile conditions, central femoral venous and arterial catheters were placed using modified PE-50 tubing. The mouse was placed in a stereotaxic frame, a 5-mm craniotomy was performed over the left parietal cortex using a dental drill, and the bone flap was removed. A brain temperature micro-probe (Physitemp, Clifton, NJ) was then inserted through the burr hole. Body temperature was also monitored by rectal probe. Immediately after craniotomy, the inhalational anesthesia was changed to 1% isoflurane and room air for 10 min before CCI and onset of HS. A mild to moderate CCI was performed with a pneumatic impactor (Bimba, Monee, IL) as previously reported with modifications. A 3-mm flat-tip impounder was deployed at a velocity of 5 m/sec and a depth of 1 mm. Brain temperature was maintained at $37^\circ \pm 0.5^\circ$C throughout the experiment. To achieve a clinically relevant level of HS, 2 mL of blood/100 g of body weight was removed via the venous catheter. This hemorrhage volume resulted in a decrease in MAP to 35–40 mm Hg. The mice remained in the HS phase for 90 min, mimicking the time between injury and the first field provision of medical attention. After the HS phase, the mice were randomized to one of four treatment groups ($n = 8$ for each group), including resuscitation with (1) LR, (2) HEX, (3) HTS, or (4) PNA.

After completing the HS phase, the mice entered the pre-hospital phase, corresponding to arrival of medical personnel and initiation of fluid resuscitation. This phase lasted 30 min. The mice were given boluses of test fluid to achieve a MAP $\geq$50 mm Hg (totaling between 1.0 and 1.5 mL). Subsequently, 0.1-mL aliquots of test fluid were administered for every minute the MAP remained less than the pre-hospital MAP target of 50 mm Hg. To simulate arrival at a definitive care setting, the mice entered the in-hospital phase. During this 30-min period, shed blood was rapidly re-infused, and a goal MAP of $\geq$70 mm Hg was maintained by the administration of additional 0.1-mL aliquots of test fluid for every minute that the MAP remained less than the in-hospital target of 70 mm Hg. During this phase inhalational anesthesia was also changed from 1% isoflurane in room air to 1% isoflurane in oxygen, which was maintained for the duration of the study. At completion of the in-hospital phase, the catheters were removed, anesthesia was discontinued, and the mice were returned to their cages. They were allowed free access to food and water, and observed for up to 7 days.

Mice were excluded from analysis by criteria defined before breaking randomization if they died during the HS phase, or if they did not reach the pre-hospital phase target MAP of $\geq$50 mm Hg with the initial boluses.

Brain temperature was monitored with a temperature probe placed in the right parietal cortex, and was maintained at $37^\circ \pm 0.5^\circ$C throughout the experiment. MAP was continuously monitored via a catheter placed in the femoral artery, and was recorded at baseline, after CCI, and every 5 min during all three phases of the study. Baseline heart rate was continuously monitored and recorded at baseline and once during each phase. Arterial blood gas, blood lactate, and glucose levels were obtained at baseline, after 30 min of shock, and at the end of the in-hospital phase.

At 7 days after the experiment, surviving mice were re-anesthetized with 4% isoflurane and killed by ice-cold saline

transcardial perfusion, followed by 10% buffered formalin phosphate perfusion and fixation of brains with subsequent embedding in paraffin at 2 weeks. Multiple 5-μm sections, 125 μm apart, from the bregma -1.82 to -2.06 were prepared from each brain, and stained with hematoxylin and eosin (H&E; Thermo Scientific, Pittsburgh, PA). Hippocampal neuronal damage was quantified with 7-day cell counts in the H&E-stained sections by a blinded evaluator (J.E.) using a Nikon Eclipse E600 microscope (Melville, NY), and Image J software (http://rsb.info.nih.gov/ij/).

Statistical analysis

Physiologic measurements and neuron counts were compared between treatment groups using one-way analysis of variance (ANOVA), and post-hoc tests with appropriate correction for multiple comparisons. All data are provided as mean $\pm$ SEM. Seven-day survival was compared between treatment groups using Fisher's exact test. Significance was determined by a p value ≤ 0.05.

Results

MAP did not differ significantly between groups at the end of the shock phase ($p = 0.31$) (Fig. 1). In contrast, the PNA and HEX groups achieved higher MAP in the pre-hospital phase than the LR or HTS groups ($p < 0.05$). MAP did not differ significantly between groups during the in-hospital phase ($p = 0.20$). The PNA and HEX groups required substantially less fluid to achieve resuscitation goals in the pre-hospital phase ($p < 0.05$) (Fig. 2). There was no difference between groups in volume required to achieve resuscitation goals in the in-hospital phase ($p = 0.63$). The PNA and HEX groups also required significantly smaller total fluid volumes to achieve resuscitation goals than the LR or HTS group ($p < 0.05$). At the end of the shock phase, arterial lactate levels in the LR, HEX, HTS, and PNA groups were 3.48 ± 1.29, 2.78 ± 0.47, 2.89 ± 0.84, and 3.40 ± 0.80 mmol/L, respectively, and did not differ significantly ($p = 0.35$). Arterial lactate levels at the end of the in-hospital phase also did not differ between groups (LR 2.00 ± 0.93 mmol/L, HEX 1.96 ± 0.78 mmol/L, HTS 2.99 ± 2.04 mmol/L, and PNA 1.50 ± 0.24 mmol/L; $p = 0.15$). Seven-day survival did not differ between groups ($p = 0.33$) (Fig. 3). H&E-stained sections of the hippocampus were also evaluated (Fig. 4). A pattern of neuronal death in the hippocampus was evident, predominantly

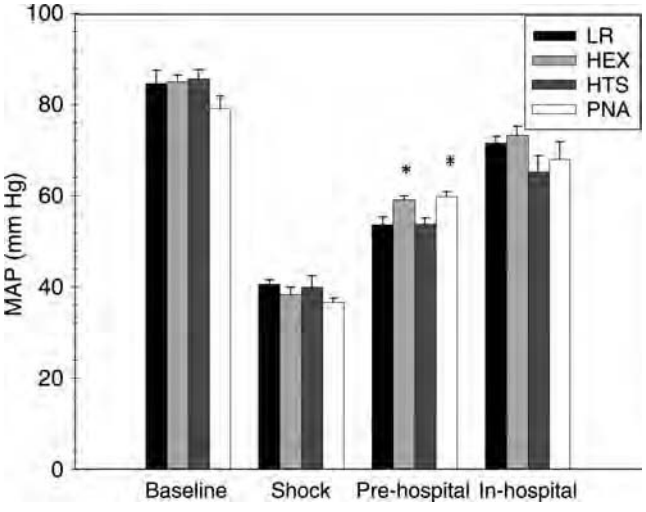

FIG. 1. Mean arterial pressure (MAP) for the experimental groups during each phase of the model (*$p < 0.05$ for the PNA and HEX groups versus the LR and HTS groups). Data are mean and SEM (n = 8 for the LR and HEX groups; n = 7 for the HTS and PNA groups; LR, lactated Ringer's solution; HEX, Hextend; HTS, hypertonic saline; PNA, polynitroxylated albumin).

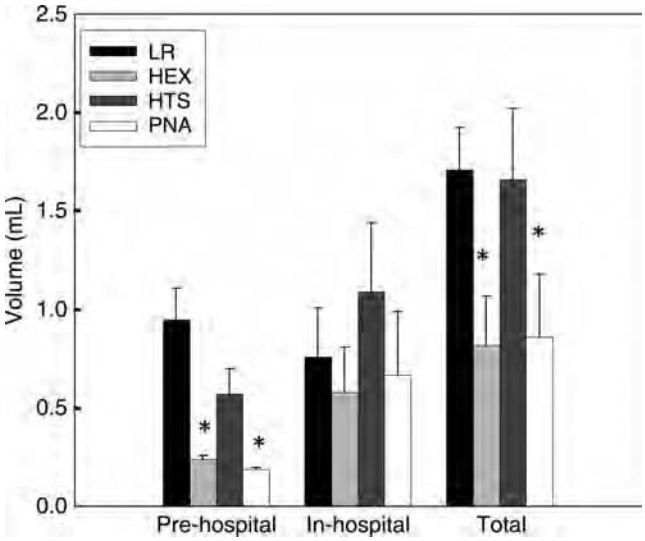

FIG. 2. Fluid requirements for the experimental groups during each phase of the model (*$p < 0.05$ for the PNA and HEX groups versus the LR and HTS groups). Data are mean and SEM (n = 8 for the LR and HEX groups; n = 7 for the HTS and PNA groups; LR, lactated Ringer's solution; HEX, Hextend; HTS, hypertonic saline; PNA, polynitroxylated albumin).

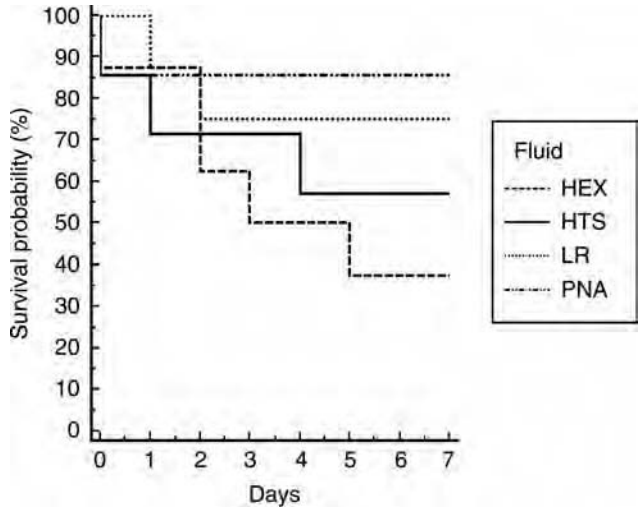

FIG. 3. Kaplan-Meier survival curve for 7-day survival probability for the four study groups.

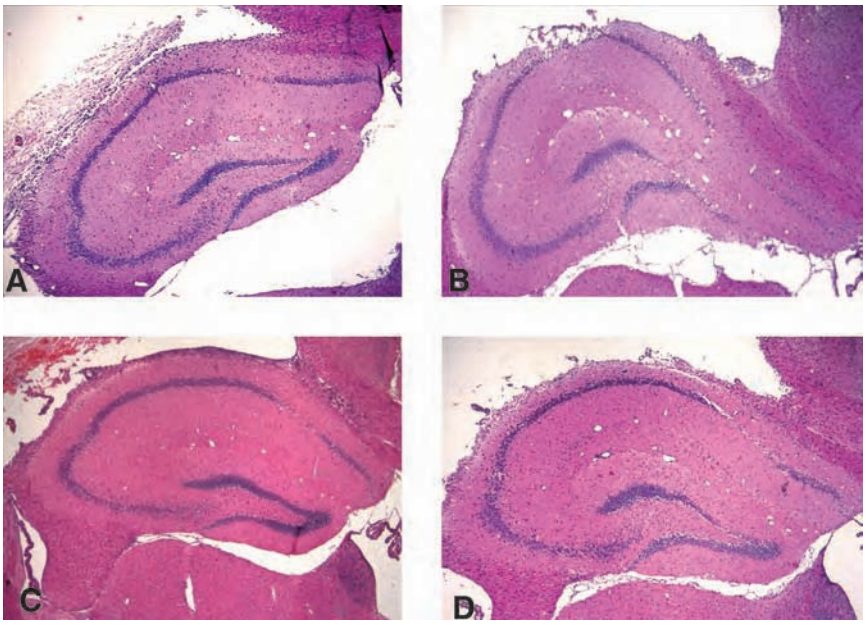

FIG. 4. Representative 40×H&E microphotographs depicting the ipsilateral hippocampus in the four study groups. (**A**) LR. (**B**) HEX. (**C**) HTS. (**D**) PNA. Pyramidal neuron loss is evident within the medial region of CA1 in all groups (LR, lactated Ringer's solution; HEX, Hextend; HTS, hypertonic saline; PNA, polynitroxylated albumin).

in the CA1 subfield, mirroring the work of Dennis and associates (2009). Evaluation of ipsilateral CA1 neuron loss (as a percentage of the contralateral hemisphere) revealed that a ~35% loss of CA1 neurons was seen at the injury level selected, which did not differ significantly between groups ($p = 0.81$) (Fig. 5). Similarly, ipsilateral CA3 neuron loss (as a percentage of the contralateral hemisphere), although much more modest than that seen in CA1, did not differ between groups ($p = 0.86$).

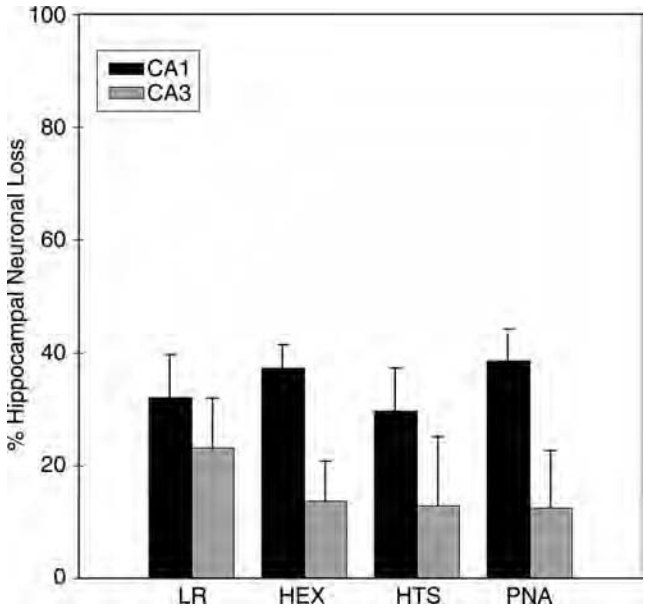

FIG. 5. Average amount of ipsilateral hippocampal neuron loss (as percentage of the contralateral hippocampal neuron count) in four study groups. Data are mean and SEM of all mice surviving to day 7 (n = 6 for the LR and PNA groups, n = 3 for the HEX group, and n = 4 for the HTS group). There was no significant difference between the four groups (LR, lactated Ringer's solution; HEX, Hextend; HTS, hypertonic saline; PNA, polynitroxylated albumin).

Discussion

Our findings show that resuscitation with the colloids PNA or HEX, in our mouse model of CCI plus HS, required less fluid volume to reach the target MAPs, and achieved and maintained higher MAPs in the pre-hospital phase, caused no adverse effects on recovery of lactate levels, and had comparable 7-day survival rates. Resuscitation with PNA or HEX also did not worsen hippocampal neuron survival versus HTS or LR. These findings are noteworthy, as the merits of resuscitation with colloids are the subject of intense debate.

Current fluid resuscitation strategies for TBI patients advise infusion of isotonic crystalloid solutions to normalize blood pressure (to maintain systolic BP > 90 mm Hg) (Badjatia et al., 2008). This recommendation has been challenged by numerous researchers who investigated the use of colloids, hypertonic fluids, vasopressors, and blood substitutes for post-TBI resuscitation. Recently, the authors of the SAFE study reported increased mortality of TBI patients treated with albumin (Finfer et al., 2004; Myburgh et al., 2007). Although no mechanism was offered to account for these findings, others have proposed the development of a dilution coagulopathy, which in the context of severe TBI worsens outcome (Billota and Rosa, 2007; Schirmer-Mikalsen et al., 2007). This subset of patients received more frequent blood transfusions early in their ICU course, but no information on the incidence of bleeding complications after enrollment in the SAFE study was provided. We did not observe excessive bleeding in our study animals. Alternatively, it is possible colloids move across the damaged blood–brain barrier, and remain trapped in brain tissue once the barrier is repaired.

After degradation to protein components, an osmotic gradient could be created, promoting edema formation and worsening brain injury (Kawamata et al., 2007). We did not evaluate brain edema in our study, but we found that resuscitation with PNA or HEX offered considerable benefit in terms of volume requirement and hemodynamic status, and did not worsen hippocampal neuronal death versus LR. The SAFE study also did not evaluate the performance of albumin in the early, acute resuscitation of TBI plus HS, making it difficult to draw comparisons with our findings.

PNA is a colloid with beneficial effects across numerous experimental paradigms. It has been shown to reduce infarct size in experimental stroke in rats (Beaulieu et al., 1998; Sugawara et al., 2001), attenuate damage in experimental myocardial ischemia in rats (Li et al., 2002) and in murine models of sickle cell crisis (Kaul et al., 2006), and highly germane to our work, it reduces mortality in experimental HS in rats (Kentner et al., 2002). These beneficial effects may result from the potent intravascular antioxidant and nitric oxide–sparing effects conferred by the covalently linked nitroxide moieties in PNA. Nitroxides have potent SOD and catalase mimetic effects, and the free nitroxide tempol is neuroprotective in experimental TBI in rats (Trembovler et al., 1999; Leker et al., 2002; Deng-Bryant et al., 2008). Beneficial effects of PNA could also be mediated by its rheologic properties (Russell et al., 1998). Any or all of these effects could contribute to the positive hemodynamic effects we observed in the PNA group. PNA and HEX could also share beneficial oncotic effects with albumin that could enhance CBF, or as a relatively small volume of the resuscitation fluid, limit edema (Tu et al., 1988a,b; Ohtaki et al., 1993). Also, the fact that mice resuscitated with PNA or HEX showed more normalized MAP levels despite significantly lower resuscitation volumes, strongly supports their putative oncotic effects.

The target MAP for resuscitation of TBI plus HS remains unclear. We chose a pre-hospital MAP target of $\geq$50 mm Hg and in-hospital MAP target of $\geq$70 mm Hg as a compromise between optimized cerebral perfusion pressure, exacerbation of bleeding that can occur in the setting of uncontrolled HS, and volume overload with pulmonary edema. Dennis and colleagues (2009) reported mortality associated with pulmonary edema in mice rapidly fluid-resuscitated to normotension after CCI plus HS. Despite modest MAP targets, the mice in the LR and HTS groups still received $\geq$60 mL/kg versus the 31–34 mL/kg given to mice in the HEX and PNA groups, which is a clinically meaningful difference. The potential contribution of aggressive fluid resuscitation to the degree of cerebral edema cannot be overlooked (Earle et al., 2007).

We analyzed neuronal survival in the hippocampus, anticipating its enhanced vulnerability to TBI plus HS. We did not find a significant difference in ipsilateral hippocampal CA1 and CA3 neuron counts between the groups. However, the colloids (PNA or HEX) did not worsen neuronal survival versus the crystalloids (HTS or LR). One might have expected deleterious effects based on the results of the SAFE study. The decision to perform neuron counts at 7 days post-insult may bias our results against any possible protective effect of PNA, as mice that died before 7 days may have had more extensive hippocampal neuron loss, and survival was numerically greatest in the PNA group.

Our study has several limitations. It would be useful to compare the effects of albumin versus PNA in our model, and we are currently examining albumin in a new protocol. However, PNA may represent a colloid quite different from albumin—with a different molecular weight, charge, and other properties. Albumin may thus not represent the perfect control. Second, our study was not powered to detect differences between treatment groups in 7-day survival. The PNA group had the highest numeric 7-day survival rate. Comparison of survival between groups yielded a $p = 0.33$ with a power of 0.5; increasing the number in each group to 15 would be needed to address this hypothesis with a power of 0.8. Third, lactate levels may not represent an optimal marker of tissue perfusion and HS in our model. Despite 90 min at a MAP $\sim$35 mm Hg (<50% of the baseline MAP), lactate levels did not increase significantly from baseline to the end of HS. Investigation of a more severe level of HS is needed. Fourth, we did not measure intracranial pressure in our mouse model. This is technically difficult in mice, and could worsen brain injury. However, we recognize that intracranial pressure is important in our understanding of changes in MAP and cerebral perfusion pressure, and it is part of our ongoing work. Fifth, we did not regulate fluid balance in the mice beyond the initial monitoring period. Free access to water could limit the duration of effects of colloids or HTS. Long-term intensive care would be required to address this issue. Finally, we did not study resuscitation in the setting of uncontrolled bleeding.

The combination of TBI plus HS is deleterious, and the ideal fluid for resuscitation of this insult has yet to be identified. We have reported that resuscitation with PNA and HEX can be accomplished with smaller volumes than with either LR or HTS, and that despite smaller volumes, higher MAPs are achieved. In addition, in contrast to the SAFE study, we did not observe adverse effects of colloids on mortality, nor did we find that colloids worsened neuronal death. Further study of resuscitation with colloids, including the antioxidant colloid PNA, with assessment of effects on both acute cerebral hemodynamics and functional outcome, is warranted in TBI plus HS.

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References

Badjatia, N., Carney, N., Crocco, T.J., Fallat, M.E., Hennes, H.M., Jagoda, A.S., Jernigan, S., Letarte, P.B., Lerner, E.B., Moriarty, T.M., Pons, P.T., Sasser, S., Scalea, T., Schleien, C.L., and Wright, D.W., and the Brain Trauma Foundation. (2008). Guidelines for prehospital management of traumatic brain injury, 2nd ed. Prehosp. Emerg. Care 12(Suppl. 1), S1–S52.

Baker, A.J., Park, E., Hare, G.M.T., Liu, E., Sikich, N., and Mazer, D.C. (2008). Effects of resuscitation on neurologic physiology after cerebral trauma and hemorrhage. J. Trauma 64, 348–357.

Beaulieu, C., Busch, E., Röther, J., de Crispegny, A., Hsia, C.J.C., and Moseley, M.E. (1998). Polynitroxyl albumin reduces infarct size in transient focal cerebral ischemia in the rat: Potential mechanism studied by magnetic resonance imaging. J. Cereb. Blood Flow Metab. 18, 1022–1031.

Billota, F., and Rosa, G. (2007). Correspondence to journal. N. Engl. J. Med. 357, 2635.

Cherian, L., Robertson, C.S., and Goodman, J.C. (1996). Secondary insults increase injury after controlled cortical impact in rats. J. Neurotrauma 13, 371–383.

Chesnut, R.M., Gautille, T., Blunt, B.A., Baldwin, N., Eisenberg, H.M., Jane, J.A., Marmarou, A., and Foulkes, M.A. (1993). The role of secondary brain injury in determining outcome from severe head injury. J. Trauma 34, 216–222.

Crookes, B.A., Cohn, S.M., Bonet, H., Burton, E.A., Nelson, J., Majetschak, M., Varon, A.J., Linden, J.M., and Proctor, K.G. (2004). Building a better fluid for emergency resuscitation of traumatic brain injury. J. Trauma 57, 547–554.

Deng-Bryant, Y., Singh, I.N., Carrico, K.M., and Hall, E.D. (2008). Neuroprotective effects of tempol, a catalytic scavenger of peroxynitrite-derived free radicals, in a mouse traumatic brain injury model. J. Cereb. Blood Flow Metab. 28, 114–126.

Dennis, A.M., Haselkorn, M.L., Vagni, V.A., Garman, R.H., Feldman, K., Bayır, H., Clark, R.S.B., Jenkins, L.W., Dixon, C.E., and Kochanek, P.M. (2009). Hemorrhagic shock after experimental brain injury in mice: Effect on neuronal death. J. Neurotrauma 26, 889–899.

Earle, S.A., de Moya, M.A., Zuccarelli, J.E., Norenberg, M.D., and Proctor, K.G. (2007). Cerebrovascular resuscitation after polytrauma and fluid restriction. J. Am. Coll. Surg. 204, 261–275.

Finfer, S., Bellomo, R., Boyce, N., French, J., Myburgh, J., and Norton, R. (2004). A comparison of albumin and saline for fluid resuscitation in the intensive care unit. N. Engl. J. Med. 350, 2247–2256.

Giri, B.K., Krishnappa, I.K., Bryan, R.M., Jr., and Robertson, C. (2000). Regional cerebral blood flow after cortical impact injury complicated by a secondary insult in rats. Stroke 31, 961–967.

Jenkins, L.W., Moszynski, K., Lyeth, B.G., Lewelt, W., DeWitt, D.S., Allen, A., Dixon, C.E., Povlishock, J.T., Majewski, T.J., Clifton, G.L., Young, H.F., Becker, D.P., and Hayes, R.L. (1989). Increased vulnerability of mildly traumatized rat brain to cerebral ischemia: the use of controlled secondary ischemia as a research tool to identify common or different mechanisms contributing to mechanical and ischemic brain injury. Brain Res. 477, 211–224.

Kaul, D.K., Liu, X.D., Zhang, X., Ma, L., Hsia, C.J., and Nagel, R. (2006). Inhibition of sickle red cell adhesion and vasoocclusion in transgenic sickle mice. Am. J. Physiol. Heart Circ. Physiol. 291, H167–H175.

Kawamata, T., Mori, T., Sato, S., and Katayama, Y. (2007). Tissue hyperosmolality and brain edema in cerebral contusion. Neurosurg. Focus 22, E5.

Kelly, M.E., Miller, P.R., Greenhaw, J.J., Fabian, T.C., and Proctor, K.G. (2003). Novel resuscitation strategy for pulmonary contusion after severe chest trauma. J. Trauma 55, 94–105.

Kentner, R., Safar, P., Behringer, W., Wu, X., Kagan, V.E., Tyurina, Y.Y., Henchir, J., Ma, L., Hsia, C.J., and Tisherman, S.A. (2002). Early antioxidant therapy with Tempol during hemorrhagic shock increases survival in rats. J. Trauma 53, 968–977.

King, D.R., Cohn, S.M., and Proctor, K.G. (2004). Changes in intracranial pressure, coagulation, and neurologic outcome after resuscitation from experimental traumatic brain injury with hetastarch. Surgery 136, 355–363.

Leker, R.R., Teichner, A., Lavie, G., Shohami, E., Lamensdorf, I., and Ovadia, H. (2002). The nitroxide antioxidant tempol is cerebroprotective against focal cerebral ischemia in spontaneously hypertensive rats. Exp. Neurol. 176, 355–363.

Li, H., Ma, L., Hsia, C.J.C., Zweier, J.L., and Kuppusamy, P. (2002). Polynitroxyl-albumin (PNA) enhances myocardial infarction therapeutic effect of tempol in rat hearts subjected to regional ischemia-reperfusion. Free Rad. Biol. Med. 32, 712–719.

Matsushita, Y., Bramlett, H.M., Kuluz, J.W., Alonso, O., and Dietrich, W.D. (2001). Delayed hemorrhagic hypotension exacerbates the hemodynamic and histopathologic consequences of traumatic brain injury in rats. J. Cereb. Blood Flow Metab. 21, 847–856.

Miller, J.D., and Becker, D.P. (1982). Secondary insults to the injured brain. J. R. Coll. Surg. Edinb. 27, 292–298.

Myburgh, J., Cooper, D.J., Finfer, S., Bellomo, R., Norton, R., Bishop, N., Lo, S.K., and Vallance, S. (2007). Saline or albumin for fluid resuscitation in patients with traumatic brain injury. N. Engl. J. Med. 357, 874–884.

Ohtaki, M., and Tranmer, B.I. (1993). Role of hypervolemic hemodilution in focal cerebral ischemia of rats. Surg. Neurol. 40, 196–206.

Prough, D.S., Whitley, J.M., Taylor, C.E., Deal, D.D., and DeWitt, D.S. (1991). Small-volume resuscitation from hemorrhagic shock in dogs: Effects on systemic hemodynamics and systemic blood flow. Crit. Care Med. 19, 364–372.

Russell, J., Okayama, N., Alexander, J.S., Granger, D.N., and Hsia, C.J. (1998). Pretreatment with polynitroxyl albumin (PNA) inhibits ischemia-reperfusion induced leucocyte-endothelial cell adhesion. Free Radic. Biol. Med. 25, 153–159.

Schirmer-Mikalsen, K., Vik, A., Gisvold, S.E., Skandsen, T., Hynne, H., and Klepstad, P. (2007). Severe head injury: control of physiologic variables, organ failure, and complications in the intensive care unit. Acta Anaesthesiol. Scand. 51, 1194–1201.

Shackford, S.R., Zhuang, J., and Schmoker, J. (1992). Intravenous fluid tonicity: effect on intracranial pressure, cerebral blood flow, and cerebral oxygen delivery in focal brain injury. J. Neurosurg. 76, 91–98.

Sugawara, T., Yu, F., Ma, L., Hsia, C.J., and Chan, P.H. (2001). Delayed treatment with polynitroxyl albumin reduces infarct size after stroke in rats. Neuroreport 12, 3609–3612.

Trembovler, V., Gallily, R., Horowitz, M., and Shohami, E. (1999). Antioxidants attenuate acute toxicity of tumor necrosis factor-alpha induced by brain injury in rat. J. Interferon Cytokine Res. 19, 791–795.

Tu, Y.K., Heros, R.C., Candia, G., Hyodo, A., Lagree, K., Callahan, R., Zervas, N.T., and Karacostas, D. (1988). Isovolemic hemodilution in experimental focal cerebral ischemia. Part 1: Effects on hemodynamics, hemorheology, and intracranial pressure. J. Neurosurg. 69, 72–81.

Tu, Y.K., Heros, R.C., Karacostas, D., Liszczak, T., Hyodo, A., Candia, G., Zervas, N.T., and Lagree, K. (1988). Isovolemic hemodilution in experimental focal cerebral ischemia. Part 2: Effects on regional cerebral blood flow and size of infarction. J. Neurosurg. 69, 82–91.

Walsh, J.C., Zhuang, J., and Shackford, S.R. (1991). A comparison of hypertonic to isotonic fluid in the resuscitation of brain injury and hemorrhagic shock. 50, 284–292.

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Selective Early Cardiolipin Peroxidation after Traumatic Brain Injury: An Oxidative Lipidomics Analysis

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Objective: Enhanced lipid peroxidation is well established in traumatic brain injury. However, its molecular targets, identity of peroxidized phospholipid species, and their signaling role have not been deciphered.

Methods: Using controlled cortical impact as a model of traumatic brain injury, we employed a newly developed oxidative lipidomics approach to qualitatively and quantitatively characterize the lipid peroxidation response.

Results: Electrospray ionization and matrix-assisted laser desorption/ionization mass spectrometry analysis of rat cortical mitochondrial/synaptosomal fractions demonstrated the presence of highly oxidizable molecular species containing $C_{22:6}$ fatty acid residues in all major classes of phospholipids. However, the pattern of phospholipid oxidation at 3 hours after injury displayed a nonrandom character independent of abundance of oxidizable species and included only one mitochondria-specific phospholipid, cardiolipin (CL). This selective CL peroxidation was followed at 24 hours by peroxidation of other phospholipids, most prominently phosphatidylserine, but also phosphatidylcholine and phosphatidylethanolamine. CL oxidation preceded appearance of biomarkers of apoptosis (caspase-3 activation, terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling–positivity) and oxidative stress (loss of glutathione and ascorbate).

Interpretation: The temporal sequence combined with the recently demonstrated role of CL hydroperoxides (CL-OOH) in in vitro models of apoptosis suggest that CL-OOH may be both a key in vivo trigger of apoptotic cell death and a therapeutic target in experimental traumatic brain injury.

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Polyunsaturated lipids have long been recognized as molecules indispensable for the structural and functional organization of the brain. Their roles as signaling molecules, as participants and coordinators of responses to physiological regulations, and as danger signals in injury, are not well known.[1,2] Because of their high susceptibility to attack by reactive oxygen species and oxidative modifications, peroxidation of polyunsaturated phospholipids is implicated in different types of brain damage. Yet, the specific functions and signaling roles of oxidized polyunsaturated phospholipids remain ill defined.[3] With the advent of mass spectrometry (MS)–based lipidomics, new perspectives in the identification of individual molecular species of phospholipids in brain functions have emerged.[4,5] However, MS analysis of *oxidized phospholipids* and their role in brain metabolic pathways, that is, oxidative lipidomics, is still poorly developed. This is mostly due to a large variety of low abundant species of peroxidized phospholipids combined with their relatively low stability during MS protocols.[6] With this in mind, we initiated oxidative lipidomics studies of brain injury. We were particularly interested in traumatic brain injury (TBI) using controlled cortical impact (CCI) model where spatial and temporal relationships between the initial damage and subsequent reactions and inflammatory/oxidative stress response are quite well-defined.[7,8]

TBI is an important contributor to the mortality and morbidity after trauma, which is the leading cause of death in infants and children.[9] TBI has been commonly associated with enhanced production of reactive oxygen species and reactive nitrogen species, antioxi-

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dant depletion, and resulting oxidative stress.[10] We reported that TBI in children caused depletion of the major water-soluble antioxidant, ascorbate, and accumulation of S-nitrosylated thiols in cerebrospinal fluid.[11,12] Among different biomarkers of oxidative stress, enhanced lipid peroxidation is one of the most prominent.[13] Accumulation of end products of lipid peroxidation has been documented in brain and cerebrospinal fluid after experimental and clinical TBI in both adults and children, respectively.[7,11,14,15] However, essential information on molecular targets, particularly specific polyunsaturated molecular species of phospholipids undergoing oxidation, is lacking.

Assessment of isoprostanes and neuroprostanes has reliably established involvement of lipid peroxidation in central nervous system injury.[16] This approach, however, defines the fatty acid composition of modified substrates but leaves the origin and identity of oxidized phospholipids sepulchered. This deficiency makes it difficult to identify causal links among lipid peroxidation, oxidative phospholipid signaling, and mechanisms of cell injury and death. Attempts have been made to define individual phospholipid classes undergoing oxidation. Cardiolipin (CL), a mitochondria-specific phospholipid, has been suggested to be a preferred oxidation substrate in neuronal injury.[17–19] However, these assessments were mostly based on the use of nonspecific fluorescent techniques utilizing nonyl acridine orange as a CL-binding reagent. The validity of this protocol has been criticized.[20,21] It has been demonstrated that cells lacking CL-synthase, completely devoid of CL, displayed a similar pattern of nonyl acridine orange responses as wild-type cells.[22] Moreover, the fluorescence response of the probe is obscured by its membrane potential–driven partition into different mitochondrial compartments and the respiration state.[20]

We recently developed an oxidative lipidomics approach that includes quantitative assessments of hydroperoxides in different major classes of phospholipids combined with their MS characterization. We discovered that oxidation of a CL catalyzed by cytochrome *c* was an early characteristic of mitochondrial response to proapoptotic challenges in vitro.[23] Accumulation of CL oxidation products was essential for the release of proapoptotic factors, including cytochrome *c*. The role of CL oxidation in the execution of the apoptotic program in vivo has not been addressed.

In this study, we applied oxidative lipidomics to analyze phospholipid oxidative modifications after TBI in vivo. We used our established CCI model in 17-day-old rats[24] in which the formation of functioning synapses in neuronal development is analogous to developmental processes in the young child.[25] By comparing oxidized molecular species and classes of phospholipids with their abundance, we identified the species of phospholipids most susceptible to peroxidation. We established that CL, predominantly its molecular species containing $C_{22:6}$, is a specific, early, and prominent target for TBI-induced oxidative injury. This suggests that CL oxidation products may signal apoptotic cell death in brain in vivo, and thus represent both a putative early biomarker of apoptosis and a key acute therapeutic target.

Materials and Methods

Controlled Cortical Impact Model

Seventeen-day-old male Sprague–Dawley rats were anesthetized with 3.5% isoflurane in O_2. The trachea was intubated with a 14-gauge angiocatheter. Anesthesia was maintained with 2% isoflurane in N_2O/O_2 (2:1). A rectal probe was inserted for temperature monitoring. The head was fixed in a stereotactic device. A craniotomy was made over the left parietal cortex with a dental drill, using the coronal and interparietal sutures as margins. A microprobe (Physitemp Instruments, Clifton, NJ) was inserted through a burr hole into the left frontal cortex to monitor brain temperature. Rats were warmed using a heat lamp to a brain temperature of 37 ± 0.5°C, isoflurane was decreased to 1%, and they were then allowed to equilibrate (30 minutes). For all studies, a 6mm metal pneumatically driven impactor tip was used; velocity was 4.0 ± 0.2m/sec, depth of penetration was 2.5mm, and duration of deformation was 50 milliseconds. After TBI, the bone flap was replaced, sealed with dental cement, and the scalp incision was closed. After a 1-hour monitoring period, rats were weaned from mechanical ventilation, extubated, and returned to their cages until further study. A mortality rate of $\leq$5% is routinely observed with this protocol by our group.[24]

ISOLATION OF CRUDE MITOCHONDRIAL/SYNAPTOSOMAL (P2) FRACTION. The crude brain mitochondrial fraction was prepared as described previously.[26] In brief, rats were perfused transcardially with ice-cold saline and then decapitated, brains (minus cerebellum) were rapidly removed, and ipsilateral pericontusional cortex was isolated and placed in 10 volumes of ice-cold 0.32M sucrose in 10mM tris(hydroxymethyl)aminomethane buffer (pH 7.4). The tissue was homogenized in a Teflon/glass homogenizer (clearance, 0.1–0.15mm) by 10 gentle up-and-down strokes. The homogenate was spun at 1,000*g* for 10 minutes to remove nuclei and cell debris. The resulting supernatant was centrifuged at 10,000*g* for 20 minutes to obtain the crude mitochondrial pellet. The final pellet was washed and centrifuged (4 minutes, 10,000*g*, 4°C). It has been shown that this protocol yields P2 fraction with relatively high content of synaptosomal mitochondria.[27] In addition, P2 fraction contains nonsynaptosomal mitochondria, synaptosomal membranes, and plasma membranes,[28] as evidenced by relatively high content of phosphatidylserine (PS; see later). We chose to use crude mitochondrial/synaptosomal fraction in the study to prevent selective isolation of only undamaged mitochondria from CCI samples.

LIPID EXTRACTION AND TWO-DIMENSIONAL HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY ANALYSIS. Total lipids were extracted from mitochondria using the Folch procedure.[29] Lipid extracts were separated and analyzed by two-dimensional high-performance thin-layer chromatography (2D-HPTLC) on silica G plates (5 × 5cm; Whatman, Florham Park, NJ).[30] The plates were first developed with a solvent system consisting of chloroform/methanol/28% ammonium hydroxide (65:25:5 vol/vol). After the plate was dried with a forced N2 blower to remove the solvent, it was developed in the second dimension with a solvent system consisting of chloroform/acetone/methanol/glacial acetic acid/water (50:20:10:10:5 vol/vol). The phospholipids were visualized by exposure to iodine vapors and identified by comparison with authentic phospholipid standards. Lipid phosphorus was determined by a micromethod.[31]

Phospholipid hydroperoxides were determined by fluorescence high-performance liquid chromatography (HPLC) of products formed in microperoxidase-11–catalyzed reaction with Amplex Red, *N*-acetyl-3,7-dihydroxyphenoxazine (Molecular Probes, Eugene, OR) as described previously.[23] Oxidized phospholipids were hydrolyzed by porcine pancreatic phospholipase A_2 (2U/μl) in 25mM phosphate buffer containing 1.0mM Ca, 0.5mM EDTA, and 0.5mM sodium dodecyl sulfate (pH 8.0 at room temperature for 30 minutes). After that 50μM Amplex Red and microperoxidase-11 (1.0μg/μl) was added and samples were incubated at 4°C for 40 min. The reaction was terminated by addition of 100μl stop reagent (10mM HCl, 4mM butylated hydroxytoluene [BHT] in ethanol). After centrifugation at 15,000*g* for 5 minutes, aliquots of supernatant (5μl) were injected into Eclipse XDB-C18 column (5μm, 150 × 4.6mm). The mobile phase was composed of 25mM NaH_2PO_4 (pH 7.0)/methanol (60:40 vol/vol). The flow rate was 1ml/min. Resorufin (an Amplex Red oxidation product) fluorescence (λ_{ex} 560nm, λ_{em} 590nm) was measured by Shimadzu LC-100AT vp HPLC system equipped with fluorescence detector (RF-10Axl) (Shimadzu, Kyoto, Japan) and autosampler (SIL-10AD vp). Data were processed and stored in digital form with Class-VP software.

Mass spectra of phospholipids were analyzed by direct infusion into a triple-quadrupole mass spectrometer (Finnigan MAT TSQ 700; ThermoFisher Scientific, San Jose, CA), a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, United Kingdom), or a quadrupole linear ion trap mass spectrometer (LXQ; ThermoFisher Scientific). After 2D-HPTLC separation samples were collected, evaporated under N_2, resuspended in chloroform/methanol 1:2 vol/vol (20pmol/μl), and used for acquisition of negative ion electrospray ionization (ESI) mass spectra at a flow rate of 5μl/min. The electrospray probe was operated at a voltage differential of −3.5 to 5.0kV in the negative or positive ion mode. Source temperature was maintained at 70°C in the case of triple-quadrupole mass spectrometers and 150°C for capillary temperature of ion trap LXQ. In addition, MS analysis was performed on a Finnigan LTQ mass spectrometer with MALDI source (ThermoFisher Scientific). Lipid samples were dissolved in chloroform/methanol 1:1 vol/vol. One microliter of lipid solution was spotted directly onto a MALDI plate and dried. A total of 0.5μl of 2,5-dihydroxybenzoic acid (25mg/ml in chloroform/methanol 1:1 vol/vol) was added to each spot as matrix. Spectra were acquired in negative ion mode using full-range zoom (m/z 500–2,000) or ultrazoom (SIM) scans. Tandem mass spectrometry (MS/MS) analysis of individual phospholipid species was used to determine the fatty acid composition. Collision-induced dissociation spectra on triple-quadrupole instruments were obtained by selecting the ion of interest and performing daughter ion scanning in Q3 at 400Da/sec using Ar as the collision gas. MS^n analysis on ion trap instruments was conducted with relative collision energy ranged from 20 to 40%, and with activation *q* value at 0.25 for collision-induced dissociation and 0.7 for pulsed-Q dissociation technique.

Clusters of signals with a mass difference of 16 known to represent two forms of glycerophospholipids, alkenyl-acyl and diacyl species, were detectable in ESI-MS spectra.[32] MS/MS fragmentation of ether-linked alkenyl (plasmalogen) species resulted in the formation of two typical product ions formed after loss of fatty acyl in sn-2 position: mono-lyso-alkenyl species and mono-lyso-acyl species. To further confirm the identity of glycerophospholipids, we exposed them to HCl fumes known to hydrolyze alkenyl-acyl glycerophospholipids to yield their lyso-acyl derivatives. The reaction products were subjected to HPTLC, and spots corresponding to glycerophospholipids were analyzed by ESI-MS. This treatment resulted in disappearance of molecular ions corresponding to molecular species of alkenyl-acyl glycerophospholipids, whereas those of diacyl phospholipids remained unchanged. MS/MS fragmentation of ether-linked alkyl-acyl-glycerophospholipids in negative mode yielded two typical deprotonated product ions formed after the loss of fatty acyl in sn-2 position: mono-lyso-alkyl species and acyl species. Product ions representing mono-lyso-alkyl species have mass differences of 14 compared with the product ions of corresponding mono-lyso-acyl species. Finally, chemical structures of glycerophospholipids were confirmed by Lipid Map Data Base using ChemDraw format (www.lipidmaps.org).

Histological Assessment

Neurodegeneration in the pericontusional area was assessed using terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) on 5μm paraffin sections cut through the dorsal hippocampus as described previously.[33]

Caspase-3 activity was measured using Caspase-Glo assay kit obtained from Promega (Madison, WI). Caspase-3 activity was expressed as the luminescence produced within 1 hour of incubation at 25°C using a ML1000 luminescence plate reader (Dynatech Labs, Chantilly, VA).

FLUORESCENCE ASSAY OF REDUCED GLUTATHIONE. Glutathione (GSH) levels were estimated in cortical homogenates using ThioGloTM-1[34] as described previously with minimal modifications.[7] GSH concentrations were determined by addition of GSH peroxidase and hydrogen peroxide to the brain homogenates, and the resultant fluorescence response was subtracted from the fluorescence response of the same specimens without addition of GSH peroxidase and hydrogen peroxide (Sigma, St. Louis, MO). A Shimadzu spectrophotometer RF-5301PC (Shimadzu, Kyoto, Japan)

was employed using 388nm (excitation) and 500nm (emission) wavelengths.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY OF ASCORBATE. Supernatant obtained after precipitation of proteins in brain homogenates by 10% trichloroacetic acid and sedimentation (2,000*g* × 10 minutes) was used for HPLC measurements, as described previously.[7]

Mitochondrial electron transport was determined by measuring the rotenone-sensitive NADH oxidase activity in an HPLC-based assay as described previously.[35,36] To provide access of NADH to synaptic mitochondria, we treated the aliquots of mitochondria fractions by nitrogen cavitation.[37] In these experiments, CL content in mitochondria samples was measured using an HPLC-based assay, as described previously.[38]

Statistical Analysis

Data are expressed as mean ± standard deviation. Brain oxidized phospholipid, GSH and ascorbate levels, and caspase-3/7 activity were compared among different groups using analysis of variance with Tukey's posttest. Rotenone-sensitive NADH:O_2 oxidoreductase activity between sham and CCI was compared using *t* test. Histological sections were assessed semiquantitatively by one of the authors masked to the study groups.

Results

Phospholipid Composition

Mitochondria, particularly synaptic mitochondria, are believed to play a pivotal role in oxidative brain injury.[39–41] Therefore, we used a mitochondria-rich synaptosomal fraction (P2) isolated from postnatal day 17 rats for our studies.

Figure 1 shows a typical 2D-HPTLC profile of major classes of phospholipids in the isolated P2 fractions. The silica spots were scraped off the plate, and the phospholipid content was quantified via the amounts of phosphatidylinositol (Table 1). TBI did not induce any significant change in the phospholipid composition of P2 fractions compared with controls except for the accumulation of lysophosphatidylcholine. This is consistent with the scale of accumulation of oxidation products not exceeding 5mol% of individual phospholipids (see later).

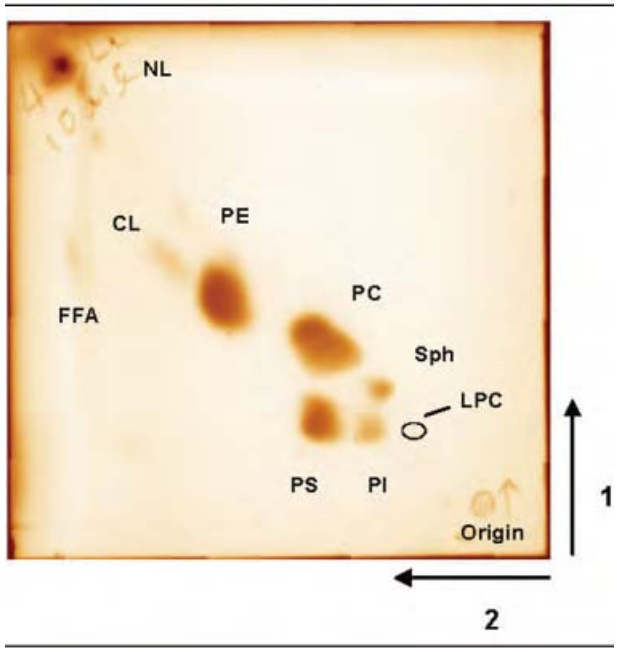

Fig 1. Typical two-dimensional high-performance thin-layer chromatography of total lipids extracted from cortical P2 fraction. CL = cardiolipin; FFA = free fatty acids; LPC = lysophosphatidylcholine; NL = neutral lipids; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; Sph = sphingomyelin.

Table 1. **Phospholipid Composition of P2 Fraction Isolated from Ipsilateral (Left) Cerebral Cortex (% of Total)**

Phospholipid Class	Control	Trauma
Cardiolipin	2.4 ± 0.3	1.9 ± 1.2
Phosphatidylethanolamine	37.7 ± 1.0	36.1 ± 0.7
Phosphatidylcholine	43.0 ± 1.8	43.3 ± 0.7
Phosphatidylserine	12.4 ± 0.7	12.3 ± 0.1
Phosphatidylinositol	3.4 ± 0.3	3.3 ± 0.4
Sphingomyelin	1.3 ± 0.7	1.6 ± 1.0
Lysophosphatidylcholine	<0.5	1.4 ± 0.8

Mass Spectroscopic Analysis of Individual Molecular Species

We further used ESI- and MALDI-MS to characterize individual molecular species of phospholipids in P2 fractions. We present detailed description of MS experiments with CL because identification of its molecular species is technically more challenging than of other phospholipids.

CL possesses two anionic charges that form both singly charged $[M-H]^-$ and doubly charged $[M-2H]^{-2}$ ions (Fig 2Aa). The identities of major CL clusters and their structures were analyzed by tandem MS using the approach that Hsu and Turk[42] described. Molecular species of brain CL were represented by at least 12 different major clusters with a variety of fatty acid residues. These included polyunsaturated arachidonic ($C_{20:4}$) and docosahexaenoic ($C_{22:6}$) (DHA) fatty acids highly susceptible to peroxidation. As an example, Figure 2Ab shows a typical MS/MS fragmentation experiment. The major CL molecular cluster of singly charged CL ion at m/z 1,550.2, which corresponds to doubly charged CL ion at m/z 774.5, yielded ions with

m/z 279, 281, 283, 303, 305, 327, and 329. These signals correspond to $C_{18:2}$, $C_{18:1}$, $C_{18:0}$, $C_{20:4}$, $C_{20:3}$, $C_{22:6}$, and $C_{22:5}$ fatty acids, which originate from at least four different CL molecular species as follows: $(C_{18:1})_1/(C_{18:0})_1/(C_{22:6})_2$; $(C_{18:0})_1/(C_{20:4})_2/(C_{22:5})_1$; $(C_{18:2})_1/(C_{20:3})_2/(C_{22:5})_1$; and $(C_{18:2})_1/(C_{18:0})_1/(C_{22:5})_1/(C_{22:6})_1$. Complete structural characterization of major CL clusters by multistage fragmentation (MS^n) using ion trap MS identified at least two isomers in each of the CL molecular species as summarized in Table 2.

These characterizations were confirmed by MALDI-MS^n. Typical ions formed during fragmentation process of CL (*a, b, a*+136, or *b*+136) were identified in MS^2 spectra.[42] Then MS^3 was performed on each of *a* or *b* ions to assign fatty acids and their positions. As an example, Figure 2Bb shows analysis of one of the precursors detected at m/z 1,472. During MALDI ionization, both $[M-H]^-$ and $[M-2H+Na]^-$ types of ions and ions of adducts with DHB matrix are formed. Three types of ions ($[M-H]^-$, $[M-2H+Na]^-$, $[M-2H+DHB+Na]^-$) and seven predominant molecular species, including four isomers, (of the *a/b* or (*a*+136)/(*b*+136) ion pairs) were identified from analysis of just one precursor in the MS^2 spectrum of the m/z 1,472 ion (see Fig 2B,b). The most dominant ion $[M-2H+Na]^-$ consisted of at least four isomers that were identified as $(C_{16:1}/C_{18:1})(C_{18:2}/C_{20:3})$; $(C_{18:2}/C_{20:4})(C_{16:0}/C_{18:1})$; $(C_{18:2}/C_{18:1})(C_{16:1}/C_{20:4})$; and $(C_{18:1}/C_{18:2})(C_{18:2}/C_{18:2})$. These isomers of CL, corresponded to doubly charged ion at m/z 724.2 (see Fig 2A). The ion $[M-H]^-$ consisted of $(C_{18:1}/C_{18:1})(C_{16:2}/C_{22:6})$ and $(C_{16:1}/C_{20:4})(C_{18:2}/C_{20:3})$. Matrix adduct ion $[M-2H+DHB+Na]^-$ corresponded to $(C_{14:0}/C_{14:0})/(C_{16:0}/C_{16:0})$.

In contrast with multiple species of CL, a single major molecular ion of PS with m/z 834 was observed using negative ionization mode (Fig 3A). PS fragmentation yielded a strong peak with m/z 747 caused by loss of the serine group. Molecular fragments with m/z 283 and 327 correspond to carboxylate anions of stearic acid ($C_{18:0}$) and DHA ($C_{22:6}$), respectively.

We used ESI-MS analysis to characterize individual molecular species of phosphatidylinositol, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin as well. The distribution of molecular species in these phospholipids, as well as characteristic fragments obtained by their fragmentation, are summarized in Table 3. Note that all major phospholipids (CL, PS, PE, and PC) contained molecular species with polyunsaturated fatty acid residues, particularly $C_{20:4}$, $C_{22:5}$, and $C_{22:6}$. These polyunsaturated fatty acids are known to be most susceptible to oxidative attack. Thus, random oxidation should cause oxidation of all of these phospholipid classes.

Phospholipid Oxidation and Identification of Individual Oxidized Molecular Species

Next, we used ESI-MS to detect and identify molecular species of phospholipids that underwent oxidation at 3 and 24 hours after CCI. Comparison of the CL spectra from ipsilateral cortex in control and CCI rats demonstrated an increased intensity of a peak at m/z 790.6. Detailed analysis of this peak demonstrated that the $[M-2H]^{2-}$ ion corresponds to multiple CL species with a dominant isomer of $(C_{18:1}/C_{22:6})(C_{22:6+OOH}/C_{18:0})$ originating from the ion at m/z 774.8 (see Fig 2C). The structural assignment of this CL-OOH product with hydroperoxy group in $C_{22:6}$ was obtained by MS^n fragmentation as described earlier (data not shown). MALDI-MS analysis confirmed this conclusion (data not shown). We performed oxidative lipidomics analysis of doubly charged species for CL because the signal intensity of doubly charged ion is higher compared with the singly charged one as shown in Figure 2A.

MS analysis of PS in the ipsilateral cortex in control and CCI rats detected presence of PS molecular species with oxidized $C_{22:6}$, PS-OOH, with m/z 866 (see Fig 3B). The intensity of this signal was higher at 24 hours

Fig 2. Typical negative ion electrospray ionization (ESI) (A) and matrix-assisted laser desorption/ionization (MALDI) (B) mass spectra of cardiolipins (CLs) obtained from cortical P2 fraction. CLs isolated by two-dimensional high-performance thin-layer chromatography (2D-HPTLC) were subjected to mass spectrometry (MS) analysis by direct infusion into mass spectrometer. (A) The identities of major molecular species in CL clusters were established by tandem MS. Shown is a typical MS/MS fragmentation experiment for a major CL molecular cluster with a single charged ion at m/z 1,550. Note the formation of ions with m/z 279, 281, 283, 303, 305, 327, and 329 corresponding to $C_{18:2}$, $C_{18:1}$, $C_{18:0}$, $C_{20:4}$, $C_{20:3}$, $C_{22:6}$, and $C_{22:5}$ fatty acids and resulting in at least four different CL molecular species as follows: $(C_{18:1})_2/(C_{18:0})_1/(C_{22:6})_2$; $(C_{18:0})_1/(C_{20:4})_2/(C_{22:5})_1$; $(C_{18:2})_1/(C_{20:3})_2/(C_{22:5})_1$; $(C_{18:2})_1/(C_{18:0})_1/(C_{22:5})_1/(C_{22:6})_1$ fatty acids (A, b). (B) Structural characterization of CL molecular species consisting of multiple isomers by Ion Trap MS^n fragmentation (B, b). MS^2 spectrum shows a singly charged CL ion at m/z 1,472; note the presence of multiple a and b fragments. (B, c) MS^3 spectrum of (a+136) *ion at m/z 829 of one of the 72:7 CL isomers. The MALDI-MS^3 spectrum of m/z 829 (1,472–829) ion confirmed the structure as $(C_{16:1})/(C_{18:1})$. All ion assignments were performed according to Hsu and Turk.[42] (C) Typical negative ion ESI mass spectra of molecular species of CL isolated from ipsilateral cortical P2 fraction after CCI. Identification of individual oxidized molecular species ($C_{22:6}$ containing CL-OOH). Tandem MS/MS experiments confirmed the structures of oxidized CL (C, a and b).*

after CCI versus control. Detailed analysis of this peak by ESI-MS demonstrated that the $[M-H]^-$ ion at m/z 866.4 corresponds to PS with dominating product of ($C_{18:0}/C_{22:6+OOH}$) originating from the ion at m/z 834 ($C_{18:0}/C_{22:6}$).

Consistent with the MS measurements, quantitative analysis of phospholipid oxidation by HPLC showed that CL underwent most robust and early (at 3 hours) oxidation after CCI (Fig 4). At this time point, no other phospholipids were oxidized. At 24 hours after

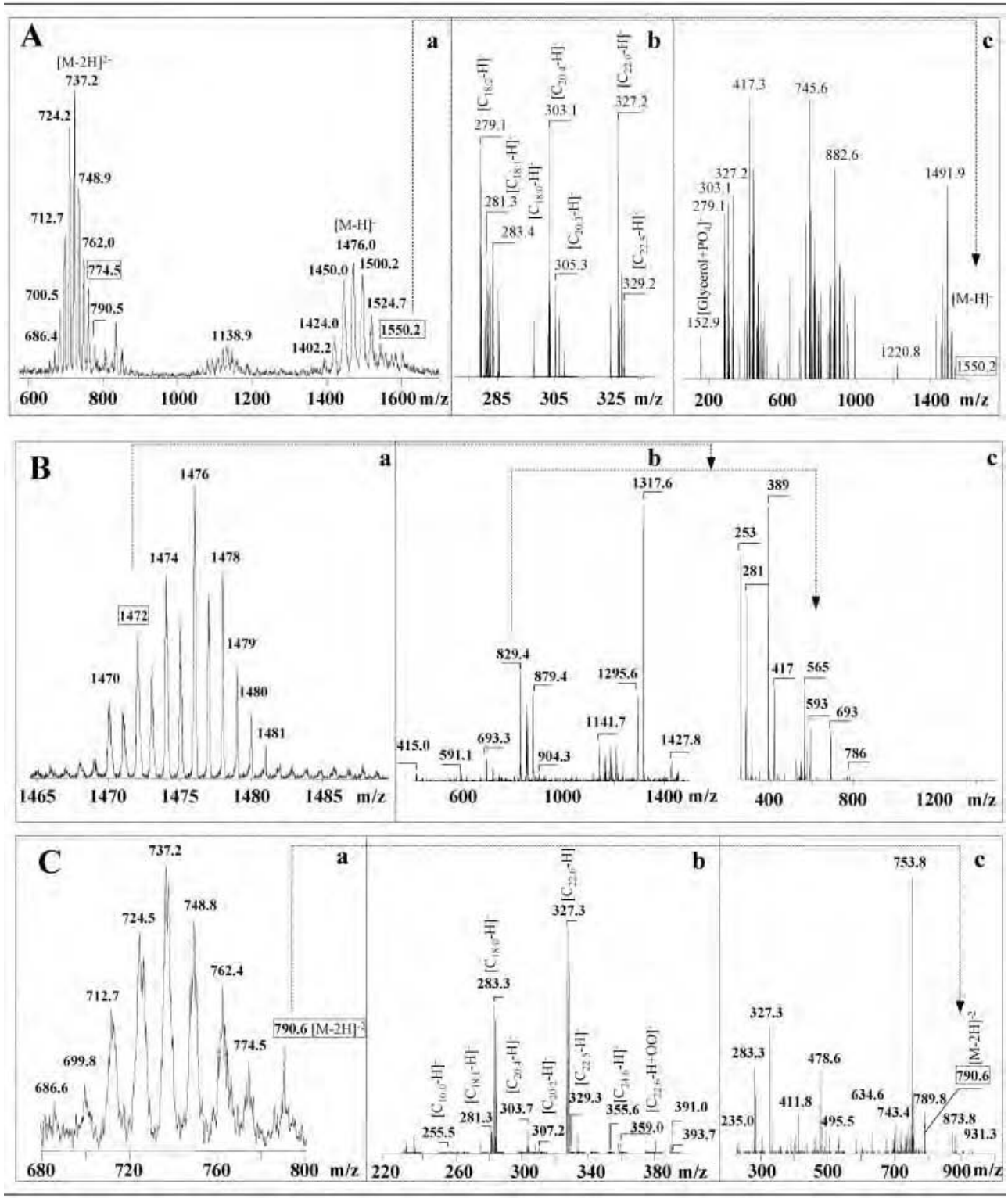

Figure 2

Table 2. **Major Cardiolipin Molecular Species from P2 Fraction of Rat Brain Cortex**

Molecular Species	m/z $[M-2H]^{-2}$	m/z $[M-H]^{-}$	Acyl Chain Composition
Cardiolipin			
Diacyl species of major clusters			*Acyl/Acyl*
68:3	686.6	1,374.2	$(C_{14:0})_1/(C_{16:1})_1/(C_{18:1})_2$
68:3	700.5	1,402.0	$(C_{16:1})_1/(C_{16:0})_1/(C_{18:1})_2$
68:2	701.9	1,404.8	$(C_{16:0})_2/(C_{18:1})_2$
70:6	711.8	1,424.6	$(C_{16:0})_1/(C_{16:1})_1/(C_{18:1})_1/(C_{20:4})_1$
70:5	712.7	1,426.4	$(C_{16:1})_1/(C_{18:2})_1/(C_{18:1})_2$
70:4	713.9	1,428.8	$(C_{16:1})_1/(C_{18:1})_3$
72:8	723.5	1,448.0	$(C_{16:1})_1/(C_{18:2})_1/(C_{18:1})_1/(C_{20:4})_1$
72:8	723.5	1,448.0	$(C_{18:2})_4$
72:7	724.5	1,450.0	$(C_{16:1})_1/(C_{18:2})_1/(C_{18:1})_1/(C_{20:3})_1$
74:8	737.2	1,476.4	$(C_{18:1})_2/(C_{18:2})_1/(C_{20:4})_1$
76:11	748.8	1,498.6	$(C_{18:2})_1/(C_{18:1})_1/(C_{20:4})_2$
76:10	749.8	1,500.2	$(C_{18:1})_2/(C_{20:4})_2$
76:9	750.8	1,502.6	$(C_{16:0})_1/(C_{18:1})_1/(C_{20:4})_1/(C_{22:4})_1$
78:15	758.9	1,518.9	$(C_{16:1})_1/(C_{18:2})_1/(C_{22:6})_2$
78:14	759.5	1,520.0	$(C_{18:2})_1/(C_{20:4})_3$
78:12	762.0	1,524.0	$(C_{18:1})_2/(C_{20:4})_1/(C_{22:6})_1$
78:10	763.8	1,528.6	$(C_{18:1})_2/(C_{20:4})_1/(C_{22:4})_1$
80:13	774.5	1,550.0	$(C_{18:1})_1/(C_{18:0})_1/(C_{22:6})_2$
80:13	774.5	1,550.0	$(C_{18:0})_1/(C_{20:4})_2/(C_{22:5})_1$
80:13	774.5	1,550.0	$(C_{18:2})_1/(C_{20:3})_2/(C_{22:5})_1$
80:13	774.5	1,550.0	$(C_{18:2})_1/(C_{18:0})_1/(C_{22:5})_1/(C_{22:6})_1$
80:12	776.0	1,553.0	$(C_{18:0})_2/(C_{22:6})_2$
82:17	784.6	1,570.2	$(C_{18:1})_1/(C_{20:4})_1/(C_{22:6})_2$
82:11	790.6	1,582.7	$(C_{18:1})_1/(C_{20:2})_2/(C_{24:6})_1$

Phospholipids are designated as follows: tetra-acyl 74:8 cardiolipin (CL), where 74 indicates the summed number of carbon atoms at both the sn-1, sn-2, and sn-1′, sn-2′ positions and :8 designates the summed number of double bonds at both the sn-1, sn-2, and sn-1′, sn-2′ positions. Possible major species are indicated as tetra-acyl $(C_{18:1})_2/(C_{18:2})_1/(C_{20:4})_1$, where 18, 18, 18, and 20 are the numbers of carbon atoms in fatty acyl chains at the sn-1, sn-2 and sn-1′, sn-2′ positions, respectively, and :1, :1, :2, and :4 are the numbers of double bonds of the sn-1, sn-2 and sn-1′, sn-2′ fatty acyl chains, respectively. These individual CL molecular species were detected by ESI as deprotonated species of CL in the negative ionization mode at m/z ratios of 737.2 and 1,476.4. These m/z values indicate ratios of mass to charge for singly charged $[M-H]^{-}$ ions and doubly charged $[M-2H]^{-2}$ ions, respectively.

TBI, a marked oxidation of PS occurred, whereas other phospholipids such as PE and PC were only slightly oxidized. Importantly, the pattern of phospholipid oxidation was nonrandom and did not follow their abundance in P2 fraction (compare with Table 1).

Cytochrome c*–Catalyzed Oxidation of Tetralinoleyl-Cardiolipin*

To investigate interaction between cytochrome *c* and CL, we performed in vitro assessments of the ability of cytochrome *c* to catalyze H_2O_2-dependent peroxidation of polyunsaturated tetralinoleyl-cardiolipin. Marked accumulation CL hydroperoxides (CL-OOH) formed in this system was detected using fluorescence HPLC protocol (Fig 5A). We then identified the major oxidation products by ESI-MS. We found that molecular species of CL containing 1, 2, 3, 4, and 5 hydroperoxy groups were generated in the course of cytochrome *c*–catalyzed reaction (see Fig 5B). In addition, several hydroxy and hydroxy-hydroperoxy derivatives of CL were detected by MS analysis. This demonstrates that nonoxidized CL undergoes oxidation to its hydroperoxides in the presence of H_2O_2. Moreover, these results also show that cytochrome *c* can uti-

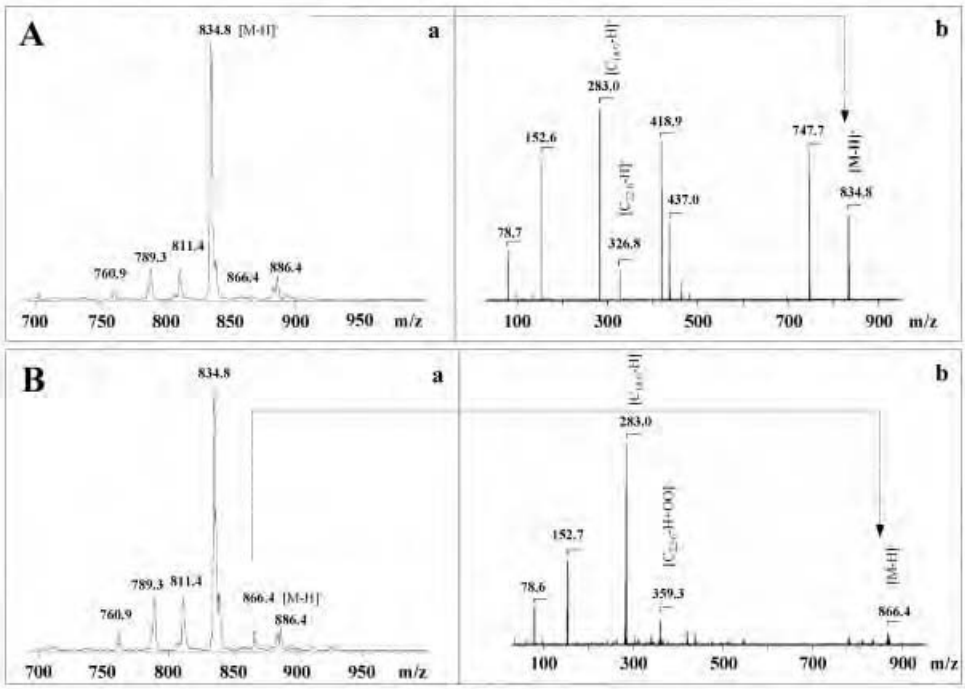

Fig 3. Typical negative ion ESI mass spectra of molecular species of phosphatidylserine (PS). Phospholipids isolated by two-dimensional high-performance thin-layer chromatography (2D-HPTLC) were subjected to mass spectrometry (MS) analysis by direct infusion into mass spectrometer. (A) Shown is a typical MS/MS fragmentation experiment for the major PS with m/z 834. PS fragmentation yielded a strong peak with m/z 747 caused by loss of the serine group. Molecular fragments with m/z 283 and 327 corresponded to carboxylate anions of stearic ($C_{18:0}$) and docosahexaenoic ($C_{22:6}$) fatty acids, respectively. (B) Typical negative ion ESI mass spectra of PS isolated from ipsilateral cortical P2 fraction after CCI. Identification of individual oxidized molecular species ($C_{22:6}$ containing PS-OOH). Tandem MS/MS experiments confirmed the structures of oxidized PS (B, a and b).

lize CL-hydroperoxides as a source of oxidizing equivalents to oxidize CL and simultaneously reduce CL-OOH to CL-OH.

Biomarkers of Cell Degeneration and Apoptosis

To determine whether there was a correspondence between phospholipid oxidation and the appearance of biomarkers of cell damage, we assessed time course of biomarkers of apoptosis. TUNEL-positivity was observed in the pericontusion cortical area at 24 hours after injury. There was no TUNEL-positive staining in sham-operated rat cortex (Fig 6A). Ipsilateral cortical caspase-3/7 activity was increased at 24 hours after CCI but not at 3 hours compared with control (see Fig 6B).

Assessments of Oxidative Stress and Mitochondrial Electron Transport Activity

GSH and ascorbate are two major water-soluble antioxidants in the brain.[43] Ipsilateral cortical GSH levels were decreased at 24 hours (10.84 $\pm$ 0.64nmol/mg protein) after CCI versus controls (14.56 $\pm$ 1.16nmol/mg protein) (Fig 7A). Ascorbate concentrations in ipsilateral cortex were lower at 24 hours in injured rats (43.02 $\pm$ 1.13nmol/mg protein) versus control rats (58.0 $\pm$ 4.74nmol/mg protein; $p < 0.05$) (see Fig 7B). Furthermore, the reductions in GSH and ascorbate levels correlated temporally with the nonspecific oxidation of phospholipids.

CL is essential for the maintenance of mitochondrial electron transport. We reasoned that CL oxidation could be associated with the loss of electron transport activity. In accord with this, we observed a significant decrease in rotenone-sensitive NADH:O_2 oxidoreductase activity at 3 hours after CCI versus control, coincident with the CL oxidation (see Fig 7C). There was no difference in cytochrome *c* oxidase subunit IV expression assessed by Western blot analysis between CCI and control (data not shown).

Discussion

Selective Early Oxidation of Cardiolipin: A Specific Apoptotic Trigger in Injured Brain?

This report presents the first detailed MS-based characterization of individual molecular species of major phospholipids in the rat cortex. Our emphasis has been placed on polyunsaturated molecular species of mitochondrial/synaptosomal phospholipids, particularly on the species containing DHA residues, as most likely targets for oxidative attack. We found that essentially

Table 3. **Major Phospholipid Molecular Species from P2 Fraction of Rat Brain Cortex**

Molecular Species	m/z $[M-H]^-$	Identified Acyl Chains
Phosphatidylinositol		
Diacyl species		*Acyl/Acyl*
34:1	835.8	$C_{16:1}/C_{18:0}$
36:4	857.8	$C_{16:0}/C_{20:4}$
38:4	885.8	$C_{18:0}/C_{20:4}$
38:3	887.8	$C_{18:0}/C_{20:3}$
40:6	909.8	$C_{18:0}/C_{22:6}$
Phosphatidylserine		
Diacyl species		*Acyl/Acyl*
34:1	760.8	$C_{16:0}/C_{18:1}$
36:1	788.8	$C_{18:0}/C_{18:1}$
38:4	810.8	$C_{18:0}/C_{20:4}$ and $C_{16:0}/C_{22:4}$
40:6	834.8	$C_{18:0}/C_{22:6}$
40:5	836.8	$C_{18:0}/C_{22:5}$
40:4	838.8	$C_{18:0}/C_{22:4}$
Phosphatidylethanolamine		
Diacyl species		*Acyl/Acyl*
38:6	762.8	$C_{16:0}/C_{22:6}$
38:4	766.8	$C_{18:0}/C_{20:4}$ or $C_{16:0}/C_{22:4}$
40:6	790.8	$C_{18:0}/C_{22:6}$
40:4	794.7	$C_{18:0}/C_{22:4}$
Alkenyl-acyl species		*Ether/Acyl*
34:1	700.8	$C_{16:0p}/C_{18:1}$
36:4	722.8	$C_{16:0p}/C_{20:4}$
38:5 or 38:6	747.8	$C_{18:1p}/C_{20:4}$ or $C_{16:0p}/C_{22:6}$
38:4	750.8	$C_{16:0p}/C_{22:4}$ or $C_{18:0p}/C_{20:4}$
38:2	754.7	$C_{18:0p}/C_{20:2}$ or $C_{18:1p}/C_{20:1}$
40:6	774.7	$C_{18:0p}/C_{22:6}$
40:4	778.8	$C_{18:0p}/C_{22:4}$
Sphingomyelin (sodium salt of molecular ion of m/z 731.7)		
Sphingoid base-acyl species		Sphingoid base/acyl
34:1	703.7	$C_{18:1}/C_{16:0}$
36:2	729.7	$C_{18:1}/C_{18:1}$
36:1	731.7	$C_{18:1}/C_{18:0}$
36:1	753.8*	$C_{18:1}/C_{18:0}$
38:1	759.8	$C_{18:1}/C_{20:0}$
42:2	813.8	$C_{18:1}/C_{24:1}$

all phospholipid classes (PC, PE, phosphatidylinositol, and CL) included $C_{22:6}$-containing species. This suggests that during a random nonenzymatic process of lipid peroxidation, phospholipids should be involved in the reaction proportionally to their abundance. Here we report that CCI-induced lipid peroxidation did not

Table 3. **continued**

Molecular Species	m/z $[M+H]^+$	Identified Acyl Chains
Phosphatidylcholine		
Diacyl species		*Acyl/Acyl*
30:0	706.7	$C_{14:0}/C_{16:0}$
32:1	732.7	$C_{16:0}/C_{16:1}$
32:0	734.7	$C_{16:0}/C_{16:0}$
34:2	758.8	$C_{16:1}/C_{18:1}$
34:1	760.7	$C_{16:0}/C_{18:1}$
34:0	762.7	$C_{16:0}/C_{18:0}$
36:3	784.7	$C_{18:1}/C_{18:2}$
36:1	788.7	$C_{18:0}/C_{18:1}$
38:6	806.7	$C_{16:0}/C_{22:6}$
38:4	810.7	$C_{18:0}/C_{20:4}$
38:2	812.7	$C_{18:0}/C_{20:2}$
40:7	832.7	$C_{18:1}/C_{22:6}$
40:4	838.7	$C_{18:0}/C_{22:4}$
Ether/Acyl species		*Ether/Acyl*
36:1	772.7	$C_{18:0p}/C_{18:1}$
32:0	718.9	$C_{16:0a}/C_{16:0}$
32:0	718.9	$C_{18:0a}/C_{14:0}$
34:0	744.9	$C_{18:0p}/C_{16:0}$
38:4	794.7	$C_{18:0a}/C_{20:4}$
38:4	794.7	$C_{16:0a}/C_{22:4}$
38:3	796.7	$C_{18:0a}/C_{20:3}$
40:7	816.7	$C_{18:1p}/C_{22:6}$
40:4	822.7	$C_{18:0a}/C_{22:4}$

p = an sn-1 vinyl ether (alkenyl- or plasmalogen) linkage; a = an sn-1 ether (alkyl-) linkage.

follow this prediction early after the impact. In contrast, only molecular species of one class of phospholipids, a $C_{22:6}$-containing CL, underwent oxidation whereas other more abundant phospholipids, particularly PC and PE, remained intact. At a later stage, however, the random character of lipid peroxidation materialized: although CL still remained the preferred peroxidation substrate, other phospholipids, particularly PS, were oxidized as well. This suggests that specific peroxidation mechanisms triggered early after CCI were followed by nonspecific random pathways at later time points.

Although lipid peroxidation has been long associated with brain injury,[10,44] its specific role in mediation of damaging pathways and signaling cascades is not well understood. Recently, signaling functions have been assigned to specific molecular species of oxidized phospholipids.[23,45] We reported that cytochrome *c*–catalyzed CL oxidation products (mostly CL-hydroperoxides [CL-OOH]) accumulate in mitochondria during apoptosis, where they play a critical role in the release of proapoptotic factors into the cytosol.[23] This enzymatic oxidation of CL might explain the specific early accumulation of CL-OOH after injury. Moreover, CL oxidation occurs early in apoptosis in nonneuronal cells preceding cytochrome *c* release, outer mitochondrial membrane permeabilization, caspase activation, and PS externalization.[23] In a separate study, we established that triggering of staurosporine-induced apoptosis in cortical neurons leads to an early and selective CL oxidation, which is not accompanied by oxidation of other more abundant phospholipids.[46] Based on these facts, it is tempting to speculate that selective CL peroxidation early after CCI reflects an initial apoptotic event in brain mitochondria. It is unlikely that CL oxidation originates from a nonspecific inflammatory re-

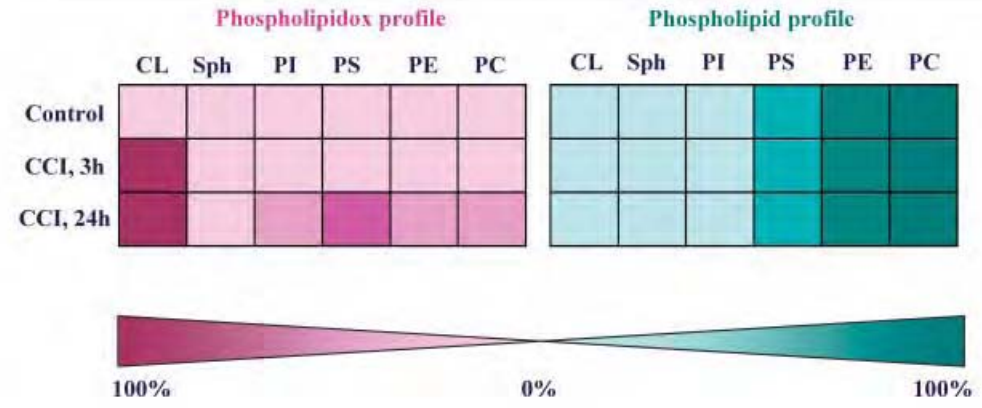

Fig 4. Comparison of the abundance of major phospholipid (PL) classes with their oxidation. Profiles of phospholipids and phospholipid hydroperoxides in control and controlled cortical impact (CCI) ipsilateral cortical P2 fractions. Phospholipid content is expressed as percentage of total phospholipids and shown in green scale. Phospholipid hydroperoxides are presented as percentage of phospholipid (pmol PL-OOH per nmol of phospholipid) and shown in purple scale. One hundred percent corresponds to 110 ± 20pmol of phospholipid hydroperoxide per nanomole of phospholipid. Cardiolipin (CL) was selectively oxidized at 3 hours after CCI, at a time point when other phospholipids were not oxidized. Phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) were oxidized at 24 hours after CCI together with CL. Sph = sphingomyelin.

sponse, which happens much later after CCI.[8] Thus, the early CL oxidation occurs in resident brain cells, likely in mitochondria-rich synaptic and dendritic neuronal projections. Despite accumulation of CL-OOH, we were not able to detect CCI-induced depletion of CL. This is because the amounts of CL-OOH formed represented only a small molar fraction of total CL. This corroborates the role of oxidized CL as an intra-

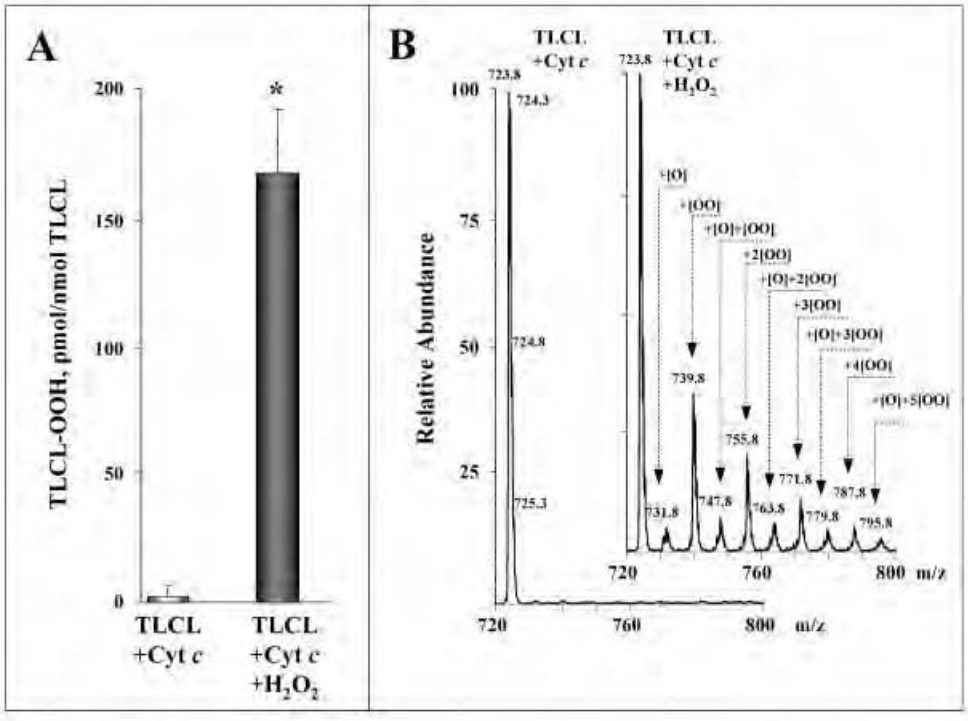

Fig 5. Characterization of cytochrome c (cyt c)–catalyzed peroxidation of tetralinoleyl-cardiolipin (TLCL) in the presence of H_2O_2. Quantitation of the amounts of TLCL-hydroperoxides by fluorescence HPLC-based assay (A). Major oxidation products were identified by ESI mass spectrometry (MS) (B). Molecular species of cardiolipin (CL) containing 1, 2, 3, 4, and 5 hydroperoxy groups were generated in the course of cyt c–catalyzed reaction. In addition, several hydroxy and hydroxy-hydroperoxy derivatives of CL were detected by MS analysis.

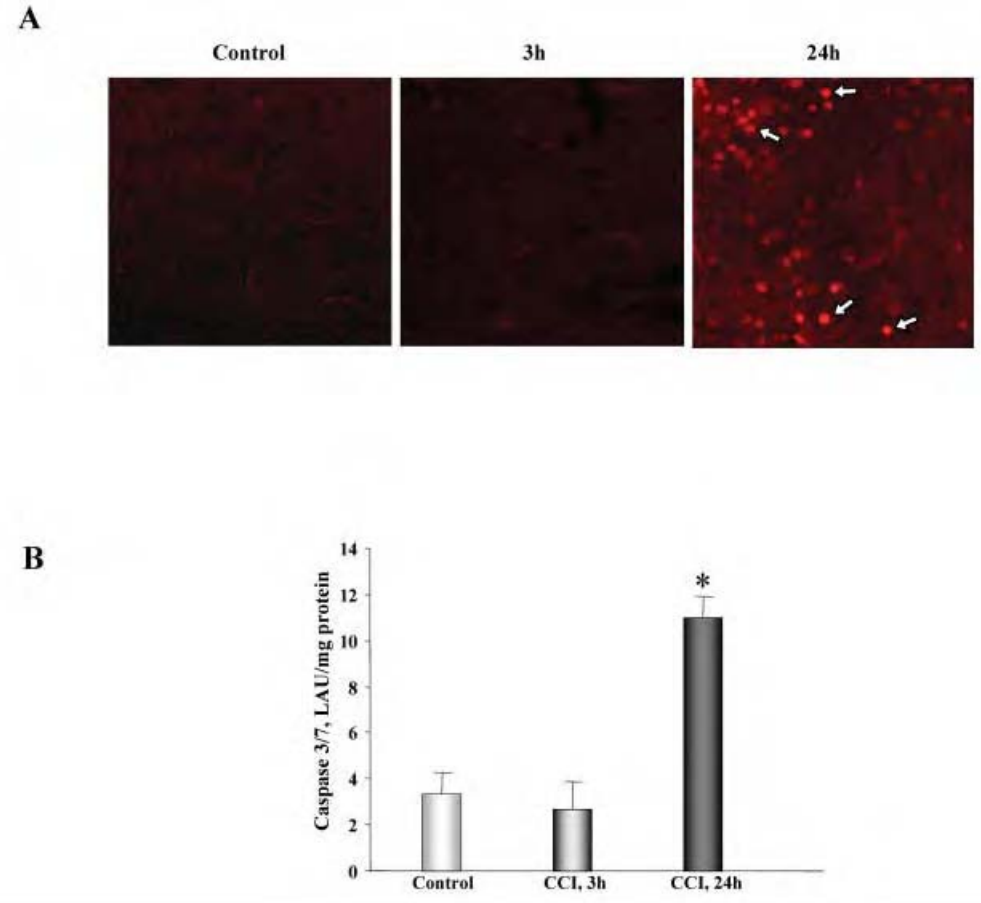

Fig 6. Analysis of cell death in the ipsilateral cortex. (A) Terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL)–positive cells were detected in the ipsilateral pericontusional cortex at 24 hours after controlled cortical impact (CCI) (arrows). *(B) Activity of caspase-3/7, measured in the ipsilateral (left) cortical tissue, was highest at 24-hour injury corroborating the histological data. (n = 5/group; mean ± standard deviation; *p < 0.05 24-hour CCI vs control and 3-hour CCI, analysis of variance).*

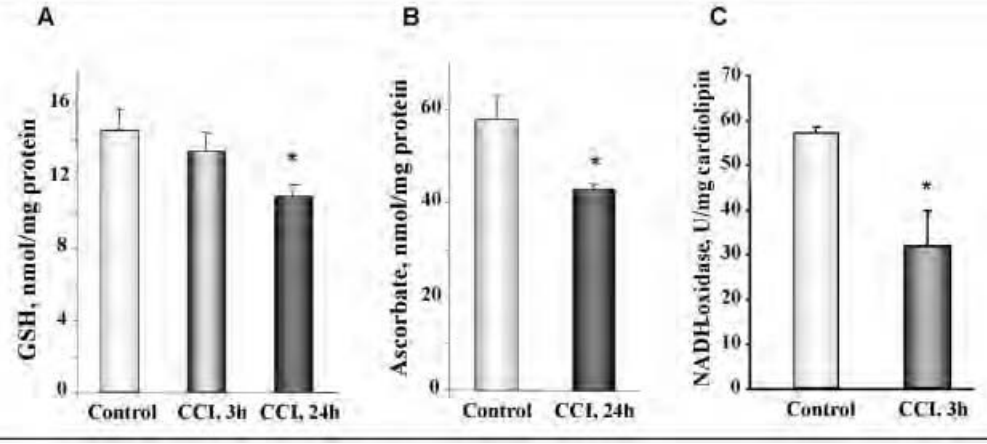

*Fig 7. Assessment of oxidative stress and mitochondrial electron transport activity. Significant decrease in reduced glutathione (GSH) (A) and ascorbate (B) levels in the ipsilateral cortical homogenates were observed at 24 hours after controlled cortical impact (CCI). (n = 5/group; mean ± standard deviation [SD]; *p < 0.05 24-hour CCI vs control for GSH [analysis of variance] and ascorbate [t test]). Rotenone-sensitive NADH:O_2 oxidoreductase activity was decreased in the ipsilateral cortical P2 fractions at 3 hours after CCI versus control. (n = 3/group; mean ± SD; *p < 0.05 3-hour CCI vs control, t test).*

cellular signaling event rather than a random phospholipid oxidation process.

Based on apparent loss of CL, previous work has suggested the involvement of CL oxidation in neuronal proapoptotic responses in vitro.[17,47] Specificity of nonyl acridine orange used in the studies is not sufficient to accurately link changes of its fluorescence characteristics with alterations of CL content and/or peroxidation.[22,48] Therefore, direct estimates of CL oxidation are necessary to prove its participation in neuronal apoptosis. Because CL is a mitochondria-specific phospholipid, our measurements of CL-OOH production rather than CL depletion provide an unequivocal evidence for CL oxidation that takes place in mitochondria. Thus, this work identifies the site (mitochondria), time (3 hours), and molecular species ($C_{22:6}$) of CL peroxidation after TBI. Finally, impairment of mitochondrial electron transport and production of reactive oxygen species are prerequisites for CL oxidation. In line with this we found that mitochondrial electron transport (NADH oxidase) activity was inhibited coincidentally with CL oxidation. This is consistent with our previous demonstration that CL oxidation acts as a switch turning off participation of cytochrome *c* in mitochondrial respiration and turning on its peroxidase function.[49]

Oxidations of Phosphatidylserine and Other Phospholipids: How Specific Are They?

We further established that PS ranked second on the scale of CCI-driven phospholipid oxidation. Again, the molecular species with $C_{22:6}$ was the one that was identifiable in MS as having PS-hydroperoxides (PS-OOH). Although we do not have direct proof for PS oxidation specifically in apoptotic cells, a later accumulation of PS-OOH (24 hours) corresponds with its known role as a signal facilitating PS externalization on the surface of apoptotic cells.[50] This interpretation is also supported by our results demonstrating that caspase-3/7 activation and appearance of TUNEL-positive cells in cortex was coincident with PS oxidation. Because mitochondria do not contain PS, oxidation of this phospholipid could predominantly occur in synaptosomal membranes, further confirming the potential signaling role of PS oxidation in its externalization.[51] At 24 hours after CCI, the most abundant phospholipids, PC and PE, also underwent oxidative modification. It is possible that PC oxidation products act as signaling molecules as well.[45]

TBI causes an increase in the level of free polyunsaturated fatty acid, particularly DHA, in injured brain regions most likely secondary to hydrolysis of phospholipids.[52] Phospholipase A_2 activity increases after TBI.[53] It is possible that oxidation of DHA ($C_{22:6}$)-containing CL and PS stimulates their hydrolysis by phospholipase A_2.[54] However, CL is not a likely source for DHA accumulation because no accumulation of lysoCLs, the products of Phospholipase A_2–catalyzed CL hydrolysis, was detected.

Mitochondrial Electron Transport Activity

Impaired brain mitochondrial function is seen after both experimental and clinical head injury.[1,55–58] Mitochondrial dysfunction begins early and may persist for days after injury. A recent study evaluated the time course of cortical mitochondrial dysfunction in adult mice after CCI and showed concomitant impairment in mitochondrial bioenergetics with accumulation of oxidative stress marker 4-hydroxynonenal as an index of global lipid peroxidation.[59] Our findings expand on these observations to the immature brain and identify one of the major contributors (CL-OOH) to overall lipid peroxidation and mitochondrial dysfunction early after injury.

CL-OOH may represent a new biomarker of oxidative injury possibly associated with an early apoptotic stage of brain damage. Clearance of apoptotic cells in the brain is mediated by oxidation and externalization of PS.[60–62] Because CL oxidation happens before peroxidation of PS, CL-OOH assessments are not likely to be masked by clearance and phagocytosis of apoptotic cells. Further developments of MS analyses can make CL-OOH evaluation in the brain feasible with an imaging protocol.[63] CL oxidation may also represent an important new target for therapeutic intervention. As a selective enzymatic reaction, CL oxidation should not be preventable by lipid antioxidants. Rather, specific disruptors of cytochrome *c*/CL interactions may be promising candidates for this purpose.

We chose to characterize individual molecular species of major phospholipids and their oxidation products after TBI in immature brain rather than adult brain for several reasons. First, trauma is the leading cause of death in children, and severe TBI is an important contributor to this mortality. Studies in pediatric TBI models represent the greatest gap in the literature as delineated by the recently published guidelines for the acute medical management of severe TBI in infants, children, and adolescents.[64] Second, several complex and interrelated pathways of programmed cell death, both caspase-dependent and caspase-independent, can occur after TBI in the developing brain.[65] The relative contribution of each might change with time after the insult and developmental stage of the animal as it has been shown for postnatal day 7 brain versus adult brain after TBI.[66] Third, greater accumulation of phospholipid hydroperoxides is expected in immature versus adult brain after TBI secondary to developmentally low activities of several antioxidant enzymes including GSH peroxidase.[67,68]

Although this study focused on acute brain injury

caused by CCI, it is possible that CL-OOH accumulation occurs in other neurological disorders leading to significant apoptotic cell death. Noteworthy, the appearance of anti-phospholipid antibodies is characteristic of a number of autoimmune diseases predisposing to or associated with brain injury.[69] Recently, it has been demonstrated that anti-CL antibodies recognize oxidized CL more effectively than CL.[70] Direct assessments of CL-OOH and its interactions with anti-phospholipid antibodies may open a new avenue in understanding their role in pathogenesis of central nervous system disorders.

Conclusion

Oxidative lipidomics is a new and exciting tool to study phospholipid oxidative modifications in vivo. Using this technique, we established that CL, specifically its molecular species containing $C_{22:6}$, is selectively oxidized early after TBI, whereas more abundant brain phospholipids remained nonoxidized at this time point. Combined with our previous data, we speculate that accumulation of CL hydroperoxides may be used as a biomarker of apoptosis in vivo that is not masked by effective clearance of apoptotic cells in the brain. Furthermore, the ability to selectively modulate CL oxidation, a critical early event in the mechanism of apoptosis, could lead to targeted therapies and ultimately improve outcome after brain injury.

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References

1. Verweij BH, Muizelaar JP, Vinas FC, et al. Mitochondrial dysfunction after experimental and human brain injury and its possible reversal with a selective N-type calcium channel antagonist (SNX-111). Neurol Res 1997;19:334–339.
2. Uauy R, Hoffman DR, Peirano P, et al. Essential fatty acids in visual and brain development. Lipids 2001;36:885–895.
3. Leitinger N. Oxidized phospholipids as triggers of inflammation in atherosclerosis. Mol Nutr Food Res 2005;49: 1063–1071.
4. Hong S, Lu Y, Yang R, et al. Resolvin D1, protectin D1, and related docosahexaenoic acid-derived products: analysis via electrospray/low energy tandem mass spectrometry based on spectra and fragmentation mechanisms. J Am Soc Mass Spectrom 2007;18:128–144.
5. Jackson SN, Wang HY, Woods AS. In situ structural characterization of glycerophospholipids and sulfatides in brain tissue using MALDI-MS/MS. J Am Soc Mass Spectrom 2007;18: 17–26.
6. Marathe GK, Harrison KA, Murphy RC, et al. Bioactive phospholipid oxidation products. Free Radic Biol Med 2000;28: 1762–1770.
7. Tyurin VA, Tyurina YY, Borisenko GG, et al. Oxidative stress following traumatic brain injury in rats: quantitation of biomarkers and detection of free radical intermediates. J Neurochem 2000;75:2178–2189.
8. Whalen MJ, Carlos TM, Kochanek PM, et al. Neutrophils do not mediate blood-brain barrier permeability early after controlled cortical impact in rats. J Neurotrauma 1999;16: 583–594.
9. Greenwald BD, Burnett DM, Miller MA. Congenital and acquired brain injury. 1. Brain injury: epidemiology and pathophysiology. Arch Phys Med Rehabil 2003;84:S3–S7.
10. Lewen A, Matz P, Chan PH. Free radical pathways in CNS injury. J Neurotrauma 2000;17:871–890.
11. Bayir H, Kagan VE, Tyurina YY, et al. Assessment of antioxidant reserves and oxidative stress in cerebrospinal fluid after severe traumatic brain injury in infants and children. Pediatr Res 2002;51:571–578.
12. Bayir H, Kochanek PM, Liu SX, et al. Increased S-nitrosothiols and S-nitrosoalbumin in cerebrospinal fluid after severe traumatic brain injury in infants and children: indirect association with intracranial pressure. J Cereb Blood Flow Metab 2003;23: 51–61.
13. Meagher EA, FitzGerald GA. Indices of lipid peroxidation in vivo: strengths and limitations. Free Radic Biol Med 2000;28: 1745–1750.
14. Hall ED, Detloff MR, Johnson K, Kupina NC. Peroxynitrite-mediated protein nitration and lipid peroxidation in a mouse model of traumatic brain injury. J Neurotrauma 2004;21:9–20.
15. Bayir H, Marion DW, Puccio AM, et al. Marked gender effect on lipid peroxidation after severe traumatic brain injury in adult patients. J Neurotrauma 2004;21:1–8.
16. Roberts LJ 2nd, Montine TJ, Markesbery WR, et al. Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. J Biol Chem 1998;273:13605–13612.
17. Kirkland RA, Adibhatla RM, Hatcher JF, Franklin JL. Loss of cardiolipin and mitochondria during programmed neuronal death: evidence of a role for lipid peroxidation and autophagy. Neuroscience 2002;115:587–602.
18. Kirkinezos IG, Bacman SR, Hernandez D, et al. Cytochrome *c* association with the inner mitochondrial membrane is impaired in the CNS of G93A-SOD1 mice. J Neurosci 2005;25: 164–172.
19. Fernandez-Gomez FJ, Gomez-Lazaro M, Pastor D, et al. Minocycline fails to protect cerebellar granular cell cultures against malonate-induced cell death. Neurobiol Dis 2005;20:384–391.
20. Garcia Fernandez MI, Ceccarelli D, Muscatello U. Use of the fluorescent dye 10-N-nonyl acridine orange in quantitative and location assays of cardiolipin: a study on different experimental models. Anal Biochem 2004;328:174–180.
21. Jacobson J, Duchen MR, Heales SJ. Intracellular distribution of the fluorescent dye nonyl acridine orange responds to the mitochondrial membrane potential: implications for assays of cardiolipin and mitochondrial mass. J Neurochem 2002;82: 224–233.
22. Gohil VM, Gvozdenovic-Jeremic J, Schlame M, Greenberg ML. Binding of 10-N-nonyl acridine orange to cardiolipin-deficient yeast cells: implications for assay of cardiolipin. Anal Biochem 2005;343:350–352.
23. Kagan VE, Tyurin VA, Jiang J, et al. Cytochrome *c* acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat Chem Biol 2005;1:223–232.

24. Jenkins LW, Peters GW, Dixon CE, et al. Conventional and functional proteomics using large format two-dimensional gel electrophoresis 24 hours after controlled cortical impact in postnatal day 17 rats. J Neurotrauma 2002;19:715–740.
25. Rice D, Barone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 2000;108(suppl 3): 511–533.
26. Weiler MH, Gundersen CB, Jenden DJ. Choline uptake and acetylcholine synthesis in synaptosomes: investigations using two different labeled variants of choline. J Neurochem 1981; 36:1802–1812.
27. Gurd JW, Jones LR, Mahler HR, Moore WJ. Isolation and partial characterization of rat brain synaptic plasma membranes. J Neurochem 1974;22:281–290.
28. Gylys KH, Fein JA, Cole GM. Quantitative characterization of crude synaptosomal fraction (P-2) components by flow cytometry. J Neurosci Res 2000;61:186–192.
29. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497–509.
30. Kagan VE, Ritov VB, Tyurina YY, Tyurin VA. Sensitive and specific fluorescent probing of oxidative stress in different classes of membrane phospholipids in live cells using metabolically integrated cis-parinaric acid. Methods Mol Biol 1998; 108:71–87.
31. Chalvardjian A, Rudnicki E. Determination of lipid phosphorus in the nanomolar range. Anal Biochem 1970;36:225–226.
32. Taguchi R, Hayakawa J, Takeuchi Y, Ishida M. Two-dimensional analysis of phospholipids by capillary liquid chromatography/electrospray ionization mass spectrometry. J Mass Spectrom 2000;35:953–966.
33. Clark RSB, Chen M, Kochanek PM, et al. Detection of single- and double-strand DNA breaks after traumatic brain injury in rats: comparison of in situ labeling techniques using DNA polymerase I, the Klenow fragment of DNA polymerase I, and terminal deoxynucleotidyl transferase. J Neurotrauma 2001;18: 675–689.
34. Langmuir ME, Yang JR, Durand RE. New thiol active fluorophores for intracellular thiols and glutathione measurement. In: Slavik J, ed. Fluorescence microscopy and fluorescent probes. New York: Plenum Press, 1996:229–234.
35. Ritov VB, Menshikova EV, Kelley DE. High-performance liquid chromatography-based methods of enzymatic analysis: electron transport chain activity in mitochondria from human skeletal muscle. Anal Biochem 2004;333:27–38.
36. Gostimskaya IS, Grivennikova VG, Zharova TV, et al. In situ assay of the intramitochondrial enzymes: use of alamethicin for permeabilization of mitochondria. Anal Biochem 2003;313: 46–52.
37. Storrie B, Madden EA. Isolation of subcellular organelles. Methods Enzymol 1990;182:203–225.
38. Ritov VB, Menshikova EV, Kelley DE. Analysis of cardiolipin in human muscle biopsy. J Chromatogr B Analyt Technol Biomed Life Sci 2006;831:63–71.
39. Abdel-Rahman A, Parks JK, Devereaux MW, et al. Developmental changes in newborn lamb brain mitochondrial activity and postasphyxial lipid peroxidation. Proc Soc Exp Biol Med 1995;209:170–177.
40. Sullivan PG, Keller JN, Mattson MP, Scheff SW. Traumatic brain injury alters synaptic homeostasis: implications for impaired mitochondrial and transport function. J Neurotrauma 1998;15:789–798.
41. Urano S, Asai Y, Makabe S, et al. Oxidative injury of synapse and alteration of antioxidative defense systems in rats, and its prevention by vitamin E. Eur J Biochem 1997;245: 64–70.
42. Hsu FF, Turk J. Characterization of cardiolipin from *Escherichia coli* by electrospray ionization with multiple stage quadrupole ion-trap mass spectrometric analysis of [M − 2H + Na]− ions. J Am Soc Mass Spectrum 2006;17: 420–429.
43. Halliwell B. Reactive oxygen species and the central nervous system. J Neurochem 1992;59:1609–1623.
44. Bazan NG, Marcheselli VL, Cole-Edwards K. Brain response to injury and neurodegeneration: endogenous neuroprotective signaling. Ann N Y Acad Sci 2005;1053:137–147.
45. Podrez EA, Poliakov E, Shen Z, et al. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. J Biol Chem 2002;277: 38503–38516.
46. Bayir H, Tyurin VA, Tyurina YY, et al. A new biomarker of early apoptosis in the brain: cardiolipin oxidation. Toxicol Sci 2006;90:410.
47. Kirkland RA, Franklin JL. Evidence for redox regulation of cytochrome C release during programmed neuronal death: antioxidant effects of protein synthesis and caspase inhibition. J Neurosci 2001;21:1949–1963.
48. Mileykovskaya E, Dowhan W, Birke RL, et al. Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces. FEBS Lett 2001;507: 187–190.
49. Basova L, Kurnikov IV, Wang L, et al. Cardiolipin switch in mitochondria: shutting off the reduction of cytochrome *c* and turning on the peroxidase activity. Biochemistry 2007;46: 3423–3434.
50. Jiang J, Serinkan BF, Tyurina YY, et al. Peroxidation and externalization of phosphatidylserine associated with release of cytochrome *c* from mitochondria. Free Radic Biol Med 2003;35: 814–825.
51. Greenberg ME, Sun M, Zhang R, et al. Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. J Exp Med 2006;203:2613–2625.
52. Homayoun P, Parkins NE, Soblosky J, et al. Cortical impact injury in rats promotes a rapid and sustained increase in polyunsaturated free fatty acids and diacylglycerols. Neurochem Res 2000;25:269–276.
53. Shohami E, Shapira Y, Yadid G, et al. Brain phospholipase A2 is activated after experimental closed head injury in the rat. J Neurochem 1989;53:1541–1546.
54. Rashba-Step J, Tatoyan A, Duncan R, et al. Phospholipid peroxidation induces cytosolic phospholipase A2 activity: membrane effects versus enzyme phosphorylation. Arch Biochem Biophys 1997;343:44–54.
55. Xiong Y, Gu Q, Peterson PL, et al. Mitochondrial dysfunction and calcium perturbation induced by traumatic brain injury. J Neurotrauma 1997;14:23–34.
56. Sullivan PG, Thompson MB, Scheff SW. Cyclosporin A attenuates acute mitochondrial dysfunction following traumatic brain injury. Exp Neurol 1999;160:226–234.
57. Okonkwo DO, Povlishock JT. An intrathecal bolus of cyclosporin A before injury preserves mitochondrial integrity and attenuates axonal disruption in traumatic brain injury. J Cereb Blood Flow Metab 1999;19:443–451.
58. Robertson CL, Puskar A, Hoffman GE, et al. Physiologic progesterone reduces mitochondrial dysfunction and hippocampal cell loss after traumatic brain injury in female rats. Exp Neurol 2006;197:235–243.
59. Singh IN, Sullivan PG, Deng Y, et al. Time course of post-traumatic mitochondrial oxidative damage and dysfunction in a mouse model of focal traumatic brain injury: implications for neuroprotective therapy. J Cereb Blood Flow Metab 2006;26: 1407–1418.

60. Kagan VE, Gleiss B, Tyurina YY, et al. A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. J Immunol 2002;169:487–499.
61. Adayev T, Estephan R, Meserole S, et al. Externalization of phosphatidylserine may not be an early signal of apoptosis in neuronal cells, but only the phosphatidylserine-displaying apoptotic cells are phagocytosed by microglia. J Neurochem 1998; 71:1854–1864.
62. De Simone R, Ajmone-Cat MA, Minghetti L. Atypical antiinflammatory activation of microglia induced by apoptotic neurons: possible role of phosphatidylserine-phosphatidylserine receptor interaction. Mol Neurobiol 2004;29:197–212.
63. Garrett TJ, Yost RA. Analysis of intact tissue by intermediate-pressure MALDI on a linear ion trap mass spectrometer. Anal Chem 2006;78:2465–2469.
64. Adelson PD, Bratton SL, Carney NA, et al. Guidelines for the acute medical management of severe traumatic brain injury in infants, children, and adolescents. Chapter 1: Introduction. Pediatr Crit Care Med 2003;4:S2–S4.
65. Felderhoff-Mueser U, Sifringer M, Pesditschek S, et al. Pathways leading to apoptotic neurodegeneration following trauma to the developing rat brain. Neurobiol Dis 2002;11:231–245.
66. Bittigau P, Sifringer M, Pohl D, et al. Apoptotic neurodegeneration following trauma is markedly enhanced in the immature brain. Ann Neurol 1999;45:724–735.
67. Fan P, Yamauchi T, Noble LJ, Ferriero DM. Age-dependent differences in glutathione peroxidase activity after traumatic brain injury. J Neurotrauma 2003;20:437–445.
68. Mavelli I, Rigo A, Federico R, et al. Superoxide dismutase, glutathione peroxidase and catalase in developing rat brain. Biochem J 1982;204:535–540.
69. Kanter JL, Narayana S, Ho PP, et al. Lipid microarrays identify key mediators of autoimmune brain inflammation. Nat Med 2006;12:138–143.
70. Horkko S, Olee T, Mo L, et al. Anticardiolipin antibodies from patients with the antiphospholipid antibody syndrome recognize epitopes in both beta(2)-glycoprotein 1 and oxidized low-density lipoprotein. Circulation 2001;103:941–946.

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Introduction

A Critical Problem Begging for New Insight and New Therapies

We dedicate this special issue of the *Journal of Neurotrauma* to the men and women of the United States military who have sacrificed their lives to defend our freedoms.

In this issue of the *Journal of Neurotrauma*, we are pleased to feature eight articles focused on the critical problem of blast-induced traumatic brain injury (TBI) and polytrauma. As a consequence of the Iraq war, blast injury has reached a new level of importance due to the high numbers of these types of injuries seen in our warfighters, and includes a wide spectrum of them, ranging from mild to severe. The pathobiology of blast-induced TBI remains poorly understood, and is complicated by many factors such as repetitive exposure and superimposed polytrauma. In the combat casualty setting, severe blast injury is a multi-system disease often involving extracerebral trauma, such as extremity injuries and burns. Blast injury is also emerging as a potential threat in civilian terrorism—such as that seen in the Madrid bombing in 2004—where combined insults such as blast-induced lung injury and TBI were observed (de Ceballos et al., 2005). We believe that the scope of articles in this issue appropriately reflects the multi-system nature of this condition in its most severe form.

We are pleased that a number of clinician-scientists and scientists working in this area have contributed either original articles or focused reviews. This issue also includes a comprehensive introductory overview on the topic by noted clinical experts Dr. Geoff Ling his and co-authors entitled "Explosive Blast Neurotrauma." Dr. Ling is the program officer overseeing the DARPA (Defense Advanced Research Projects Agency) PREVENT (Preventing Violent Explosive Neurotrauma) blast research program, which is focused on blast-induced TBI, and trying to shed light on some of the many mysteries that remain to be elucidated about this condition. Dr. Ling and his team provided neurosurgical and neurocritical care to our warfighters in Iraq, and thus impart in this review vital first-hand knowledge to the research community that reads *Journal of Neurotrauma* about this problem as it is currently being treated in theatre in Iraq (Ling et al., 2009). This should prove to be an extremely important article for those working in our field. We must link the field, the bedside, and the bench, if those of us in the neurotrauma research community are going to discover the answers to the many questions that remain in this field, and to help develop new therapies to treat this condition. The importance of this guiding principle is emphasized by Dr. Joseph Long and his colleagues at the Walter Reed Army Institute of Research in a report entitled "Blast Overpressure in Rats: Recreating a Battlefield Injury in the Laboratory," which shows that chest protection can be an important determinant of the severity of air-blast injury to the rat brain (Long et al., 2009).

We were also very pleased to receive manuscripts addressing a number of highly relevant topics to this condition, such as the seminal characterization of a model of blast-induced TBI in large animals by Dr. Richard Bauman and his colleagues in the PREVENT program titled "An Introductory Characterization of a Combat-Casualty-Care Relevant Swine Model of Closed Head Injury Resulting from Exposure to Explosive Blast" (Bauman et al., 2009). This group is currently carrying out critical studies of this problem by developing a unique model of munitions blast injury in swine, focusing on biomechanical, pathobiological, neuropathological, and neurologic consequences.

Dr. Svetlov and the group from Banyan Biomarkers, Inc., have provided us with an interesting report that addresses the important interface between experimental blast TBI models and biomarkers of brain injury that helps provide further insights into this field (Svetlov et al., 2009). We also received a related article by Dr. Denes Agoston and his group at the Uniformed Services University of the Health Sciences, that discusses proteomic applications in blast and non-blast TBI with a special focus on edema, inflammation, and neuronal death cascades (Agoston et al., 2009). Reviews are also included from Dr. YungChia Chen and colleagues on the utility of *in-vitro* systems to study blast-induced TBI (Chen et al., 2009), and from Drs. Douglas DeWitt and Donald Prough on experimental models of combined TBI plus secondary insults (DeWitt and Prough, 2009). These investigators are experts in their respective areas, and their articles address aspects of blast-induced TBI that are extremely relevant to those who work in our field. How to model blast injury *in vitro* is an important question, and polytrauma and secondary insults are so common in this condition that the neurotrauma research community must learn more about the impact of these insults, both on primary injury, and on the evolution of secondary damage (Okie, 2005; Gawande, 2004; Aschkenasy-Steuer et al., 2005; de Ceballos et al., 2005). Finally, here you will find a description of a new model of combined TBI and hemorrhagic shock in mice that will allow the future use of mutant mice in experiments to help examine some of the putative mechanisms of secondary damage, neuroprotection, and repair in these types of combined insults (Dennis et al., 2009), as heretofore such investigations

have largely focused on optimizing fluid resuscitation in large animal models.

There have been several recent reviews and seminal reports on blast-induced TBI that have addressed various aspects of this condition (Bhattacharjee, 2008; Hoge et al., 2008; Armonda et al., 2006; Okie, 2005; Gawande, 2004; Aschkenasy-Steuer et al., 2005; de Ceballos et al., 2005; Dennis and Kochanek, 2007), but many questions remain to be answered. For example, does blast-induced TBI differ from the spectrum of injuries seen in conventional civilian TBI? What experimental models of blast injury are best to study the human condition? Similarly, how accurately do our established TBI models such as controlled cortical impact or fluid percussion model blast-induced TBI in humans, and what are their shortcomings? What is the best way to test the different therapies for blast-induced TBI? What are the optimal approaches to mild versus severe blast-induced TBI, or single versus repetitive exposures? In this context, new knowledge about blast-induced TBI may allow us re-examine current treatments for conventional civilian TBI in a new light. For example, have we underestimated the role of vasospasm in conventional civilian TBI, based on the findings recently reported by Armonda and associates (2006) for blast-induced TBI? We believe the articles in this issue will help to build upon the seminal initial reports and reviews, and will provide new insights and stimulate new avenues of investigation into this crucial area of treating our warfighters, as well as the civilian population.

We also thank Drs. Claudia Robertson, Douglas DeWitt, Samuel Tisherman, and Hülya Bayir for the helpful suggestions they made during the preparation of this issue. Finally, I know that we speak for all of the authors whose work appears here in thanking Dr. John Povlishock, Editor-in-Chief, for giving us the opportunity to assemble this superb collection of articles for *Journal of Neurotrauma*.

Finally, in this issue, Dr. Povlishock has also included a special article on combination theraples for TBI authored by Drs. Susan Margulies, Ramona Hicks, and the Combination Theraples for TBI Workshop Leaders. This important article identifies key objectives and recommendations for the ultimate development of combination therapeutic approaches for TBI, a strategy that is likely to be critical to success in the many complexities addressing this condition.

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References

Agoston, D.V., Gyorgy, A., Eidelman, O., and Pollard, H. (2009). Proteomic biomarkers for blast neurotrauma: Targeting cerebral edema, inflammation, and neuronal death cascades. J. Neurotrauma 26, 901–911.

Armonda, R.A., Bell, R.S., Vo, A.H., Ling, G., DeGraba, T.J., Crandall, B., Ecklund, J., and Campbell, W.W. (2006). Wartime traumatic cerebral vasospasm: recent review of combat casualties. Neurosurgery 59, 1215–1225.

Aschkenasy-Steuer, G., Shamir, M., Rivkind, A., Mosheiff, R., Shushan, Y., Rosenthal, G., Mintz, Y., Weissman, C., Sprung, C.L., and Weiss, Y.G. (2005). Clinical review: the Israeli experience: conventional terrorism and critical care. Crit. Care 9, 490–499.

Bauman, R., Ling., G., Tong, L., Januszkiewicz, A., Agoston, D., Delanerolle, N., Kim, Y., Ritzel, D., Bell, R., Ecklund, J., Armonda, R., Bandak, F., and Parks, S. (2009). An introductory characterization of a combat-casualty-care relevant swine model of closed head injury resulting from exposure to explosive blast. J. Neurotrauma 26, 841–860.

Bhattacharjee, Y. (2008). Shell shock revisited: Solving the puzzle of blast trauma. Science 319, 406–408.

Chen, Y.C., Smith, D.H., and Meaney, D.F. (2009). In-vitro approaches for studying blast-induced traumatic brain injury. J. Neurotrauma 26, 861–876.

de Ceballos, J.P., Turegano-Fuentes, F., Perez-Diaz, D., Sanz-Sanchez, M., Martin-Llorente, C., and Guerrero-Sanz, J.E. (2005). 11 March 2004: The terrorist bomb explosions in Madrid, Spain—an analysis of the logistics, injuries sustained and clinical management of casualties treated at the closest hospital. Crit. Care 9, 104–111.

Dennis, A.M., and Kochanek, P.M. (2007). *Pathobiology of Blast Injury. Yearbook of Intensive Care and Emergency Medicine.* J.L. Vincent (ed)., Springer-Verlag: Berlin, pps. 1011–1022.

Dennis, A.M., Haselkorn, M.L., Vagni, V.A., Garman, R.H., Janesko-Feldman, K., Bayır, H., Clark, R.S.B., Jenkins, L.W., Dixon, C.E., and Kochanek, P.M. (2009). Hemorrhagic shock after experimental traumatic brain injury in mice: Effect on neuronal death. J. Neurotrauma 26, 889–899.

DeWitt, D., and Prough, D. (2009). Blast-induced brain injury and posttraumatic hypotension and hypoxemia. J. Neurotrauma 26, 877–887.

Gawande, A. (2004). Casualties of war—military care for the wounded from Iraq and Afghanistan. N. Engl. J. Med. 352, 2471–2475.

Hoge, C.W., McGurk, D., Thomas, J.L., Cox, A.L., Engel, C.C., and Castro, C.A. (2008). Mild traumatic brain injury in U.S. soldiers returning from Iraq. N. Engl. J. Med. 358, 453–463.

Ling, G., Bandak, F., Armonda, R., Grant, G., and Ecklund, J. (2009). Explosive blast neurotrauma. J. Neurotrauma 26, 815–825.

Long, J.B., Bentley, T.L., Wessner, K.A., Cerone, C., Sweeney, S., and Bauman, R.A. (2009). Blast overpressure in rats: Recreating a battlefield injury in the laboratory. J. Neurotrauma 26, 827–840.

Okie, S. (2005). Traumatic brain injury in the war zone. N. Engl. J. Med. 19, 2043–2047.

Svetlov, S.I., Larner, S.F., Kirk, D.R., Atkinson, J., Hayes, R.L., and Wang, K.K.W. (2009). Biomarkers of blast-induced neurotrauma: Profiling molecular and cellular mechanisms of blast brain injury. J. Neurotrauma 26, 913–921.

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CORTICAL SPREADING DEPRESSION AFTER TRAUMATIC AND PENETRATING INJURY TO THE HUMAN BRAIN

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Introduction: Cortical spreading depression (CSD) is a propagating wave of tissue depolarization that causes profound changes in cellular water and ion distributions, cerebral blood flow and metabolism, and BBB permeability, and represents a potential 'silent' cause of secondary deterioration. In an ongoing cooperative study, we investigate 1) characteristics and incidence of CSD after traumatic brain injury, 2) the pathophysiologic significance of CSD, and 3) factors influencing its occurrence. Methods: Electrode strips were placed subdurally in 23 patients with GCS 3-14 (median: 7) who received craniotomy for treatment of brain injury (n = 8 motor vehicle accident, 7 fall, 6 assault, 2 gunshot). Electrocorticographic (ECoG) recordings were made in four bipolar channels for 3.1 (2.2, 4.1) days (median; 1st, 3rd quartile), beginning 0.9 (0.8, 1.9) days after injury, and analyzed as in Fabricius et al. (Brain 129: 778 – 790, 2006). Patients were sedated and administered phenytoin prophylactically. Results: In 14/23 patients (61%), a total of 130 CSDs occurred at a frequency of 3.0 (1.9, 4.6) events per day. The timing of CSDs showed a biphasic U-shaped distribution, with peak incidence during the first and seventh day post-injury. The speed of CSD propagation through cerebral cortex was 2.4 (1.2, 3.0) mm/min and maximum depression of 0.5-70 Hz ECoG activity during CSD was 66% (56, 76). Depression lasted 5.9 (4.5, 8.9) min for single CSDs and was often longer on channels closest to the injury. Total depressed periods represented up to 10% of individual recordings. Two patients had recurrent CSDs, evidenced by propagating changes in near-DC slow potentials, in otherwise electrically silent tissue that corresponded to hypodensity on CT scans. Patients with parenchymal damage or intracerebral hemorrhage had a greater likelihood (12/16, 75%) of exhibiting CSD than those with diffuse swelling or sub-dural hemorrhage only (2/7, 29%; $p = 0.06$). GCS at admittance was positively correlated with CSD rate ($R^2 = 0.21$; $p = 0.03$), but there was no effect of age, sedative (morphine, propofol, midazolam), or cause of injury on incidence. Discussion: With the limited spatial sampling and recording durations used here, a 61% incidence of CSD suggests that transient, spontaneous tissue depolarizations are extremely common after traumatic brain injury and may approach ubiquity if particular pathologies are present. CSD with electrical silence or prolonged ECoG depression might serve as marker of progressive ischemia in injury penumbra and represents a potential target or indicator for therapy.

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EXACERBATION OF NEURONAL INJURY BY HEMORRHAGIC SHOCK AFTER MILD CONTROLLED CORTICAL IMPACT IN MICE

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Blast injury from improvised explosive devices (IEDs) results in a spectrum of TBI severities often accompanied by shock from hemorrhage, extracerebral trauma, and/or burns. This mechanism of injury has become a critical aspect of military and civilian trauma from terrorist attacks (1). Despite many studies of secondary injury using hypoxemia after TBI in rodent models, few studies have investigated the effect of hemorrhagic shock (HS) on neuronal death after experimental TBI; no such studies have been carried out in mice.

HYPOTHESIS: We hypothesized that cortical damage after mild controlled cortical impact (CCI) would be markedly exacerbated by a level of HS that otherwise produces no cerebral consequences (2).

METHODS: C57BL6J male mice were anesthetized with 1% isoflurane, brain temperature and mean arterial blood pressure (MAP) were monitored, and mild CCI (3 m/sec, 0.5mm) was delivered followed by either continued anesthesia for 90 min (mild CCI only) or 60 min of volume controlled HS (2.5cc/100g withdrawal) (mild CCI + HS). In the mild CCI + HS group, 6% hetastarch (the standard US military resuscitation fluid) was infused to restore MAP of 60 mmHg for 30 min, mimicking field resuscitation and evacuation. This was followed by re-infusion of shed blood and normalization of hemodynamics, approximating hospital resuscitation. Mice were sacrificed at 24 h and neuronal damage in cortex was assessed with TUNEL.

RESULTS: HS produced a MAP of 41.73 +/− 8.15 mmHg in the mild CCI + HS group, while MAP was 90.0 +/− 2.29 mmHg in the mild CCI only group. Our preliminary results reveal that neuronal injury was ~ two-fold greater in cortex in mild CCI + HS versus mild CCI alone (282 +/− 55 vs 126 +/− 154 TUNEL positive neurons, respectively).

CONCLUSIONS: Despite using a very mild CCI and a level of HS that produces no neuronal injury in normal brain, we observed a marked exacerbation of neuronal injury in the combined insult. This suggests a critical need to define optimal fluid and neuroprotective resuscitation strategies in the setting of blast injury and other forms of TBI accompanied by HS.

Gawande. NEJM. 2004; 2. Carrillo et al. J Trauma. 1998.

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INTRACRANIAL PRESSURE MONITORS PLACED BY NON-NEUROSURGEONS

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THE NITRIC OXIDE DONOR, NOC-12, ENHANCED GAP JUNCTION COMMUNICATION BETWEEN VASCULAR SMOOTH MUSCLE CELLS IN VITRO

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Introduction: Gap junctions (GJ) contribute to vasodilation and vasoconstriction and, perhaps, to cerebral vascular regulatory mechanisms such as autoregulation (1-3). Nitric oxide (NO) is a potent systemic and cerebral vasodilator. We explored the effects of the NO donor, NOC-12, on GJ between smooth muscle *in vitro*.

Methods: Rat vascular smooth muscle cells (A7r5, ATCC) were maintained in tissue culture flasks in DMEM supplemented with 10% fetal bovine serum and transferred to collagen-coated flex plates (Flexcell International). Cells were exposed to 10μM or 100μM NOC-12 30 minutes before GJ communication was assayed using fluorescence recovery after photobleaching (FRAP)(4). Cells were loaded with 5-carboxyfluorescein diacetate and intracellular fluorescence was monitored using confocal fluorescence microscopy (Zeiss LSM510). FRAP was expressed as percent of baseline fluorescence.

Results: FRAP was 17.7% of baseline in the untreated group. Treatment with 10μM or 100μM NOC-12 increased FRAP to 37.0% or 37.5%, respectively ($p < 0.01$ vs untreated group).

Discussion: Our results of NO-mediated increases in GJ coupling suggest that NO plays a role in the regulation of intercellular coupling in vascular smooth muscle cells. NO increased briefly and then decreased significantly and the NOS substrate L-arginine improved CBF and vasodilatory responses to hypercapnia after traumatic brain injury (TBI) (5-7), suggesting that TBI decreases CBF and impairs vasodilation by reducing NO synthesis or inactivating NO. Our results that TBI impaired GJ communication between vascular smooth muscle cells (8) and that NOC-12 significantly increased GJ communication, suggest that trauma-induced reductions in NO lead to reduced GJ coupling that contributes to cerebral hypoperfusion and impaired cerebral vascular reactivity after TBI. 1. Stroke 2003;34:544; 2. AJP 1989;256:H838; 3. AJP 2002;283:H2177; 4. Nat Neurosci 1998;1:494; 5. J Neurophysiol 2000;83, 2171; 6. J Neurotrauma 1997 14: 223; 7. J CBF & M 2000; 20:820; 8. Anes 2005;103:A166

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EFFECT OF HEMORRHAGIC SHOCK ON CEREBRAL BLOOD FLOW IN EXPERIMENTAL TRAUMATIC BRAIN INJURY: MAGNETIC RESONANCE IMAGING ASSESSMENT.

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Introduction: Hemorrhagic shock (HS) worsens outcome after traumatic brain injury (TBI). Controversy remains regarding ideal pre-hospital blood pressure and fluid resuscitation strategies for combined TBI and HS. Perfusion Magnetic Resonance Imaging (MRI) using arterial spin-labeling is an attractive method to serially assess the effect of HS and resuscitation after experimental TBI. **Hypothesis:** Cerebral hypoperfusion after controlled cortical impact (CCI) is exacerbated by a level of HS that otherwise has no effect on CBF. **Methods:** Male C5BL6 mice (n=13), anesthetized with isoflurane, underwent CCI (5m/sec, 1.0 mm) followed by 60 min of volume-controlled HS (2.0ml/100g) (CCI+HS), or continued anesthesia (CCI alone). Naive and HS groups were also studied. In CCI+HS and HS alone, mice were resuscitated with hetastarch for 30 min (pre-hospital) followed by return of shed blood for 60 min(definitive care). Serial coronal MRI CBF images through the injury were obtained at 4.7 Tesla during HS, pre-hospital and definitive care phases. CBF was quantified in ml/100g/min. **Results:** MABP decreased from 102.7 mmHg±3.0 to 51.7±4.4 during HS, and recovered to 75.7±0.3 after definitive care (both $p<0.05$ vs baseline). During all phases after TBI, CBF ipsilateral to CCI was reduced in CCI+HS and CCI alone groups vs naïve (79±14, 118±15 vs 203±5 $p<0.05$). CBF was not reduced by HS alone (216±28,NS). Surprisingly, contralateral to injury, CCI+HS produced a marked decrease in CBF vs all other groups during HS (CCI+HS 118±10 vs HS 203±5, CCI 182±13, naïve 217±11, $p<0.05$). Resuscitation did not restore CBF to control levels ipsilateral to injury. **Conclusions:** CCI with or without HS results in a local CBF reduction. HS after CCI produces a global CBF reduction suggesting a diffuse autoregulation impairment. Our model and MRI application provides a powerful tool to study novel approaches to optimal CBF resuscitation after TBI. Support US Army PR054755 W81XWH0610247

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GENDER- AND CELL COMPARTMENT-DEPENDENT POLY-ADP-RIBOSYLATION AFTER TRAUMATIC BRAIN INJURY IN JUVENILE RATS.

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Introduction: Traumatic brain injury (TBI) is a leading cause of morbidity and mortality in children. Energy failure and apoptosis are important contributors to secondary brain injury and both may be mediated by over-activation of the ubiquitous enzyme poly(ADP-ribose) polymerase (PARP). Inhibition of PARP genetically or pharmacologically improves outcome in multiple models of TBI. Further, a role for both nuclear and mitochondrial PARP has been implicated in neuronal death (J Biol Chem 2003;278:18426-33), and this effect may be gender dependent (J Neurochem 2004;90:1068-75). **Hypothesis:** Organelle- and gender-specific expression of poly (ADP-ribose)[PAR]-modified proteins (a surrogate biomarker of PARP activation) will be observed in brain in postnatal day (PND) 17 rats after TBI. **Methods:** Anesthetized PND 17 male and female rats were subjected to controlled cortical impact (CCI) to the left parietal cortex. Rats were killed at 24 and 48 h, naive rats were used as controls (n=3/group). Injured cortex and hippocampus were removed and separated into nuclear(n)-, mitochondrial(m)- and cytosol(c)- enriched protein fractions. PAR-modified proteins were identified using Western Blots and semiquantified by densitometry. **Results:** After CCI, n-PAR was increased only in males (*P≤0.05 vs. control, #P≤0.05 vs. females, 2-way ANOVA). m-PAR and c-PAR were increased in both genders after CCI; m-PAR elevation was sustained to 48h in males only (*P<0.05 vs. control, #P<0.05 vs. females). **Conclusions:** PAR-modified proteins are increased in a gender- and cell compartment-specific manner after TBI, suggesting that males may benefit more from PARP inhibition vs. females. Further study is warranted to determine if the effects of PARP inhibitors on neurological outcome are gender-dependent. Support: NS38620/HD40686/NS30318

PAR-modified proteins (ROD, Mean +/- SE)

	Naive male	24h male	48h male	Naive female	24h female	48h female
Nuclear	7.0 +/-0.3	10.2 +/-0.7*#	11.3 +/-0.4*#	6.3 +/-0.9	6.6 +/-0.5	7.1 +/-0.4
Mitochondrial	2.4 +/-0.04	4.5 +/-0.3*	3.5 +/-0.2*	3.4 +/-0.3	4.8 +/-0.3*	2.9 +/-0.3
Cytosolic	3.9 +/-0.3	18.7 +/-1.9*	14.4 +/-1.9*	4.3 +/-0.2	19.3 +/-1.6*	15.1 +/-2.2*

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MYOCARDIAL POTASSIUM CHANNEL REMODELING: A CANDIDATE MECHANISM FOR SUDDEN DEATH IN EPILEPSY.

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Introduction: Potassium (K+) channels play a critical role in the regulation of excitability. We previously have shown an activity-dependent decrease in levels of the K+ channel, Kv4.2 in brain from epileptic rats. Kv4.2 channels regulate neuronal excitability through dampening action potential amplitude postsynaptically; thus, a downregulation of Kv4.2 is expected to increase excitability. Kv4.2 channels also regulate the duration of the myocyte action potential in rodents. Remodeling of rodent myocardial Kv4.2 channels following seizures has not previously been evaluated. **Hypothesis:** The studies presented here test the hypotheses that there is a downregulation of myocardial Kv4.2 channels in epileptic rodents and that Kv4.2 deficiency is associated with increased sensitivity to convulsant stimulation. **Methods:** Western blotting with a Kv4.2 antibody was performed using myocardial membranes prepared from epileptic rats and sham controls. Convulsant stimulation was performed in Kv4.2 knockout and wildtype littermate mice and the time to onset of seizures and status epilepticus was assessed. **Results:** Myocardial Kv4.2 levels were decreased in epileptic compared to sham control animals ($p<0.05$; n=3). As expected Kv4.2 knockout mice have a decreased latency to first seizure and status epilepticus compared to wildtype mice ($p<0.05$). Surprisingly, we also observed that 100% of knockout mice (n=6) and only 24% of wildtype mice (n=8) died in response to convulsant stimulation. **Conclusions:** Our findings suggest that there is activity-dependent remodeling of Kv4.2 channels in both hippocampus and myocardium in a rodent model of epilepsy. Furthermore, our studies reveal that Kv4.2 deficiency is associated with a lower seizure threshold and increased risk for sudden death with convulsant stimulation. These findings support the possibility that ion channel remodeling occurs in epilepsy and may be a risk factor contributing to sudden death in epilepsy. Supported by: NIH,NINDS and Partnership for Pediatric Epilepsy Research

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METABOLIC BENEFITS OF SURFACE COUNTERWARMING DURING THERAPEUTIC TEMPERATURE MODULATION.

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Introduction: Therapeutic temperature modulation (TTM) in brain injured patients commonly results in shivering, which is associated with increases in oxygen consumption (VO2) and resting energy expenditure (REE). Treatment of shivering with sedatives is associated with adverse effects and non-pharmacological interventions to treat shivering are desired. **Hypothesis:** Surface counter warming (CW) with an air circulation blanket set at 42 C can provide beneficial control of shivering and improve the metabolic profile during TTM in brain injured patients. **Methods:** Ventilated brain injured patients undergoing TTM with automated surface and intravascular devices were prospectively studied with continuous indirect calorimetry (IC). All were administered buspirone, acetaminophen, and CW during TTM. IC was performed with >= 10 min with CW, >=10 minutes off CW, and >=10 min with return of CW. Shivering severity during IC was scored using the Bedside Shivering Assessment Scale (BSAS). REE, VO2, and carbon dioxide production (VCO2) were determined by IC. Expected energy expenditure (EE) was calculated using the Harris – Benedict Equation. Hypermetabolic index (HMI) was calculated from the ratio of REE to EE. **Results:** 45 IC tests were performed in 30 patients between January – July 2006. 59% were women, with mean age 61 +/- 13 years and body mass index (BMI) of 26.4 +/- 4.1. During 71 % of IC tests patients had signs of shivering (BSAS >0, n = 32). REE (2233.7 +/- 997 v. 2507.9 +/- 1303.5 kcal/24h, $P < 0.001$), VO2 (339.5 +/- 155.5 v. 379.5 +/- 203.5 mL/min, $P<0.001$), VCO2 (222.9 +/- 94.5 v. 250.5 +/- 120.4 mL/min, $P<0.001$), and HMI (1.53 +/- 0.6 v. 1.71 +/- 0.7, $P<0.001$) all increased with removal of CW. 89% of patients had > 1 point increase in BSAS without CW. Return of CW was associated with a reversal in the increases in REE, VO2, VCO2, and BSAS. **Conclusions:** Surface counter warming provides beneficial control of shivering and improves the metabolic profile in patients undergoing TTM.

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EFFECT OF HEMORRHAGIC SHOCK ON NEURONAL DEATH AFTER EXPERIMENTAL TRAUMATIC BRAIN INJURY IN MICE.

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Introduction: Traumatic brain injury (TBI) resulting from blast injury is often complicated by hemorrhagic shock (HS) in victims of terrorist attacks with improvised explosive devices. Most studies of HS after experimental TBI have focused on intracranial pressure; few have explored the effect of HS on neuronal death after TBI and none have been done in mice. **Hypothesis:** Neuronal death in hippocampus is exacerbated by HS after experimental TBI in mice. **Methods:** C57BL6J male mice (n=16) were anesthetized with isoflurane, brain temperature and mean arterial blood pressure (MAP) were monitored, and controlled cortical impact (CCI) (5 m/sec, 1mm depth) was delivered followed by either continued anesthesia (CCI only) or either 60 or 90 min of volume controlled HS (2.0cc/100g)(60CCI+HS and 90CCI+HS). In the CCI+HS groups, hetastarch was used to restore MAP to 50 mmHg for 30 min, (field resuscitation) followed by return of shed blood (in-hospital resuscitation). Neuronal damage in hippocampus was assessed on d 7 in H&E-stained brain sections by a blinded evaluator scoring CA1 and CA3 regions (0-no damage, 1-rare, 2-<25% affected, 3-25%-50% affected, 4-severe neuronal loss, 5-infarction). **Results:** HS reduced MAP from a baseline value of ~85 mmHg to ~35 mmHg during HS in both 60CCI+HS and 90CCI+HS ($p<0.05$ vs CCI only). MAP recovered to ~70 mmHg after in-hospital resuscitation. 90 min of HS dramatically exacerbated hippocampal damage vs either CCI alone or 60min CCI+HS. Mean scores for CA1 in CCI, 60CCI+HS and 90CCI+HS were 1.52±0.44, 1.27±0.45, and 3.25±1.39, respectively, $p<0.05$. In CA3 scores were 1.60±0.55, 1.45±0.4, and 2.93±1.18, respectively, $p<0.05$. There was no difference between CCI alone and 60CCI+HS. All mice with scores of 4 or 5 were in the 90CCI+HS group. **Conclusions:** Our data suggest a critical time window for the exacerbation of neuronal death by HS after experimental TBI and may have important implications for blast injury victims in austere environments where definitive management of injuries is delayed. Support: US Army PR054755 W81XWH0610247

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CORRELATION OF INITIAL CEREBROSPINAL FLUID MAGNESIUM LEVEL AND OUTCOME IN SEVERE TRAUMATIC BRAIN INJURY.

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Introduction: Experimental brain injury studies have demonstrated an important role for magnesium [Mg^{++}]in neurologic outcome, with low levels being associated with poor outcome, and replacement therapy improving outcome[1]. However, the correlate in clinical trials is lacking in traumatic brain injury (TBI) studies and inconclusive in other neurologic diseases[2]. **Hypothesis:** We hypothesized that a low initial cerebrospinal fluid (CSF) [Mg^{++}] following a severe TBI would predict poor neurologic outcome, as measured by 6-month Glasgow Outcome Scale (GOS) score. **Methods:** Under an IRB-approved protocol, demographic data, laboratory results, CSF samples and 6-month GOS scores were collected prospectively in severe adult TBI patients, with a Glasgow Coma Scale (GCS) score ≤ 8, age 16-70 years, and an extraventricular drain inserted for intracranial pressure management. Total [Mg^{++}] was measured in triplicate utilizing a quantitative colorimetric assay (BioAssay Systems, Hayward, CA)on the initial CSF sample taken within 24 hours of hospital admission. Binary logistic regression was performed on initial CSF [Mg^{++}] of < or > 2.68 mg to dichotomized GOS (poor outcome: 1-3, and favorable outcome: 4-5). **Results:** 44 patients (mean ± SD age 35.1 ± 16.1; 9 female, 35 male; GCS 5.7 ± 1.5)were assessed, with the initial CSF [Mg^{++}] median of 2.68 ± .73 mg. Using this median [Mg^{++}], patients with an initial [Mg^{++}] of > 2.68 mg were found to have a greater likelihood of having an unfavorable outcome (OR 7.74, CI 1.01-59.20, $p=.049$). In patients with a poor outcome, CSF and serum [Mg^{++}] were highly correlated ($r=.61$, $p=.001$). **Conclusions:** These preliminary findings in human TBI suggest that, contrary to experimental animal studies, a high admission CSF [Mg^{++}] is associated with poor neurologic outcome. Blood brain permeability changes may explain the correlation of CSF and serum [Mg^{++}]in poor outcome patients. Additional research is aimed at understanding this alteration of intracranial [Mg^{++}]homeostasis in TBI patients. [1] *J Neurochem 73(1), 1999;* [2] Lancet 363, 2004. *Support: NS 30318*

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TRAUMATIC BRAIN INJURY IN IMMATURE RATS CAUSES EARLY AND SUSTAINED ALTERATIONS IN CEREBRAL METABOLISM.

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Introduction: Traumatic Brain Injury (TBI) is the leading cause of long-term disability in children in the United States (CDC). Studies have shown profound alterations in cerebral metabolism after TBI, but pediatric data is limited, with few studies performed <48 h after TBI. **Hypothesis:** We hypothesize that metabolic changes occur early (<24h) post TBI and persist for many days. **Methods:** Immature rats (PND 16-17; n=8) underwent controlled cortical impact (CCI) to the left parietal cortex. Brains were removed at 4h, 24h, and 7d after TBI, separated into left (L, injured) and right (R, control) hemispheres and rapidly frozen. Metabolites were extracted with perchloric acid, and proton nuclear magnetic resonance spectra were obtained. Spectra were analyzed for N-acetyl-aspartate (NAA), Lactate (Lac), total Creatine (Cr), Choline (Cho), and metabolite ratios determined. Sham rats (n=6) underwent surgery without impact. **Results:** There was no difference between L and R at any time in the sham rats. The NAA/Lac ratio was decreased (~15%) at all times in the injured cortex ($p<0.05$ v. R at 4h, 24h, 7d). This reduction was due to altered Lac as evidenced by increased Lac/Cr at 4h (Lac/Cr: Left=1.31± 0.11, Right=1.05± 0.08, $p<0.05$) and 24h (Lac/Cr: Left=1.32 ± 0.07, Right=1.11± 0.02, $p<0.05$), reflecting increased glycolysis and/or decreased oxidative metabolism. At 7d NAA was decreased (NAA/Cr: Left=0.68± 0.01, Right=0.74± 0.02, $p<0.05$), indicating loss of neuronal or mitochondrial integrity. Increased Cho/Cr ratio at 7d is consistent with gliosis in the injured cortex. **Conclusions:** Metabolic derangements begin early (<4h) after TBI in immature rats and are sustained for at least 7d. Understanding the pathologic alterations in cerebral metabolism after TBI could identify novel targets for neuroprotection following TBI in children. Support NIH K08NS42805 & University of Maryland Dept of Pediatrics

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AN OBSERVATIONAL STUDY OF BLEEDING EVENTS (BE) ASSOCIATED WITH LOW-DOSE UNFRACTIONATED HEPARIN (LDUFH) IN A NEUROLOGIC INTENSIVE CARE UNIT (NSICU).

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Introduction: While DVT and pulmonary embolism (PE) remain a significant cause of morbidity and mortality in critically ill patients, clinicians are often reluctant to initiate early LDUFH prophylaxis due to the fear of BE. **Hypothesis:** Describe the BE incidence and associated risk factors related to the use of prophylactic LDUFH in NSICU patients. **Methods:** Patients admitted to the NSICU over a 9-month period were included. A major BE was defined as clinically overt blood with a decrease in Hgb by >2gm/dL and/or a transfusion of 2U PRBC; minor BE were all other BE. **Results:** A total of 181 patients were included, the majority admitted with hemorrhagic stroke(41%) or traumatic brain injury(27%). BE occurred in 14.9%(n=25) patients (41% major) 3[1-25] days after initiation of LDUFH with the majority gastrointestinal. Demographics were similar except for APACHE II: BE 15[2-24], NBE 10[0-36](p=0.03). The only difference in baseline labs was glucose, which was increased in patients with BE(p=0.02). LDUFH prophylaxis was administered in 93% of patients with BE and 84% with NBE, primarily at a dose of 5000U q12h (82%)(p=0.38). LDUFH was initiated within 24hrs of hospital admission in 84% of patients in both groups. The overall incidence of DVT and PE was 2%. Significant risk factors for BE in patients who received LDUFH are summarized below. Logistic regression revealed two independent predictors of BE; NSAID use and mechanical ventilation. **Conclusions:** The early use of LDUFH prophylaxis was not associated with an increased BE risk in this NSICU cohort even in patients with initial intracranial bleeding. Identified risk factors for bleeding may indicate that patients are at risk from other factors besides LDUFH prophylaxis.

	No BE (%)	BE (%)	p
Surgical intervention	37	63	0.03
Mechanical ventilation	39	80	<0.01
Presence of ICP monitor	20	44	0.02
Use of NSAIDs	26	52	0.02

ASL-MRI Assessment of the Effect of Hemorrhagic Shock on Cerebral Blood Flow After Experimental Traumatic Brain Injury in Mice

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INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of traumatic death in the US. Morbidity and mortality resulting from TBI are greatly increased by secondary insults such as hemorrhagic shock (HS). The combination of TBI and HS has taken on great importance related to military and civilian casualties from blast injury in combat and terrorist attacks. Hypotension worsens the outcome for patients with TBI, which is thought to be due to secondary ischemia caused by cerebral hypoperfusion. Aggressive fluid resuscitation is recommended to maintain mean arterial blood pressure (MABP), but in patients with uncontrolled hemorrhage increasing MABP can increase blood loss and reduce survival [1]. Currently, there is controversy over how to best treat patients with TBI and uncontrolled hemorrhage. TBI produces CBF reductions that are generally localized to the injury site. The aim of this study was to examine the effect of HS on regional CBF after controlled cortical impact (CCI) in mice.

MATERIALS AND METHODS

Male C57Black/6J mice (11-15 wks of age) were divided into one of four groups for MRI assessment, naïve, CCI, HS and CCI + HS. Mice were anesthetized with isoflurane in N_2O:O_2 (1:1), intubated and mechanically ventilated; then femoral arterial and venous catheters were surgically placed. The mouse CCI model is used as previously described [2] with minor modifications [3]. Animals were placed in a stereotaxic holder and a temperature probe was inserted through a burr hole into the left frontal cortex. The parietal bone was removed for trauma. Once brain temperature reached 37˚C and was maintained at this temperature for 5 minutes, a vertically directed CCI was delivered at 5.0m/sec with a depth of 1.0mm. The bone flap was replaced, sealed with dental cement and the incision closed. CCI was followed by 60 min of volume controlled HS (2 mL/100 g) (CCI + HS), or continued anesthesia (CCI), or just 60 min of volume controlled HS (HS). In CCI + HS and HS only groups, mice were resuscitated with Hextend until MABP was >50 mm Hg (pre-hospital) followed 30 min later by the return of shed blood (definitive care). Perfusion images were obtained during the shock, pre-hospital and definitive care periods.

MR studies were performed on a 4.7-Tesla, 40 cm bore Bruker AVANCE system, equipped with a 15 cm diameter shielded gradient insert and a home-built saddle-type RF coil. For all imaging experiments, FOV = 4 cm and slice thickness = 2 mm. Maps of T_{1obs} [4] were generated from a three-parameter exponential fit to a series of spin-echo images with variable *TR* (*TR* = 8000, 4300, 2300, 1200, 650, 350, 185, 100 msec, 2 averages, 128 x 70 matrix). Perfusion spin-echo images were acquired in duplicate using the arterial spin-labeling technique [5] (*TR/TE* = 2000/10, 20, 30, summation of 3 echoes, 2 averages, 128 x 70 matrix) with labeling applied ± 2 cm from the imaging plane. The spin labeling efficieny (α) [6] was determined in each study with gradient echo images with spin-labeling applied at ± 6 mm (*TR/TE* = 100/9.6 msec, 45˚ flip angle, 8 averages, 256 x 256 matrix). Body temperature was maintained at 37 ± 0.5 ˚C using warm air, regulated with a rectal temperature probe. Prior to, and after each MRI study, $PaCO_2$, PaO_2, MABP, HR and rectal temperature was recorded.

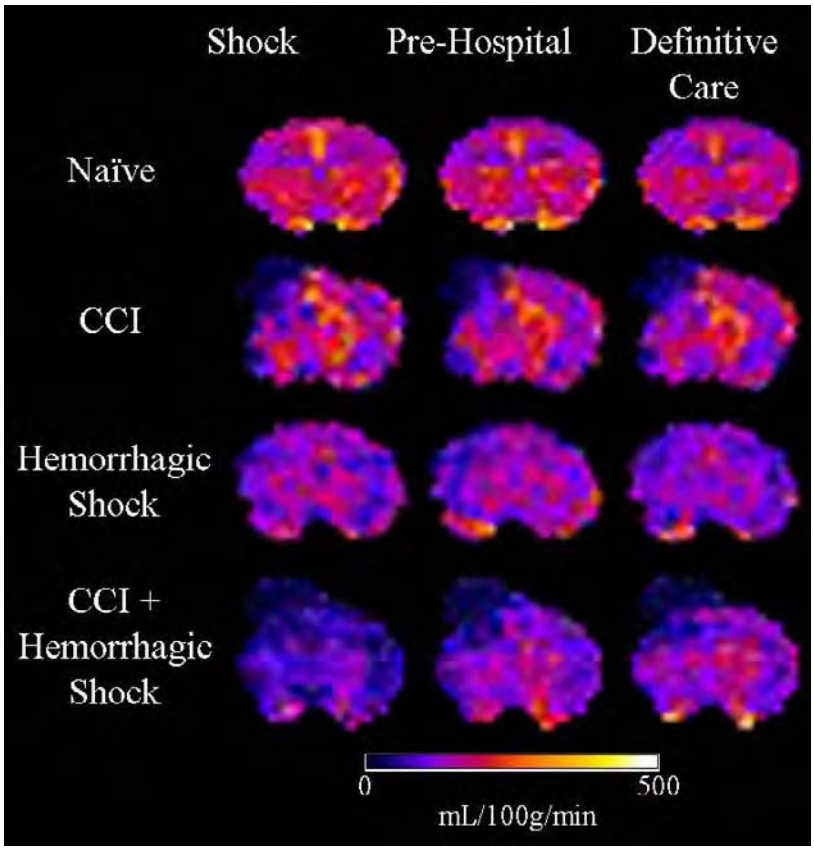

Figure 1: Representative CBF maps of mice brains with and without trauma (CCI) and with and without hemorrhagic shock, during the shock period (2 mL/100 g volume controlled blood withdrawal), pre-hospital period (resuscitation with Hextend), and definitive care period (return of shed blood).

RESULTS AND DISCUSSION

Figure 1 shows representative CBF maps for all groups. After CCI, CBF was significantly lower in the ipsilateral hemisphere, cortex and hippocampus during all phases. For HS alone mice CBF was generally lower than naïve mice but this was not significant. During the shock phase the CCI + HS mice displayed a dramatic global CBF reduction. After resuscitation, CBF the contralateral hemisphere partially recovered, but not to naïve levels, during the prehospital and definitive care phases. CBF in the ipsilateral hemisphere remained significantly decreased vs naïve mice throughout the entire experiment and, resuscitation did not restore contusional CBF. . Our data support the occurrence of a diffuse autoregulatory impairment during HS after TBI. Impaired oxygen delivery by HS superimposed upon increased metabolic demands and disturbed microcirculation after TBI, may magnify the damage, producing poor outcomes. This model using MRI provides a powerful tool to study novel approaches to optimize CBF resuscitation after TBI.

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REFERENCES

1. Novak L, Shackford SR, Bourguignon P, Nichols P, Buckingham S, Osler T and Sartorelli K. *J. Trauma* **47**, 834-844 (1999).
2. Smith DH, Soares HD, Pierce JS, Perlman KG, Saatman KE, Meaney DF, Dixon CE, and McIntosh TK. *J Neurotrauma* **12**, 169-178 (1995).
3. Whalen MJ, Carlos TM, Dixon CE, Schiding JK, Clark RS, Baum E, Yan HQ, Marion DW, and Kochanek PM. *J Neurotrauma* **16**, 299-309 (1999).
4. Hendrich KS, Kochanek PM, Williams DS, Schiding JK, Marion DW and Ho C. *Magn. Reson, Med.* **42**, 673-681 (1999).
5. Detre JA, Leigh JS, Williams DS and Koretsky AP. *Magn. Reson. Med.* **23**, 37-45 (1992).
6. Zhang W, Williams DS and Koretsky AP. *Magn. Reson. Med.* **29**, 416-421 (1993).

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EXACERBATED GLIAL RESPONSE IN THE AGED MOUSE HIPPOCAMPUS FOLLOWING CONTROLLED CORTICAL IMPACT INJURY

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Introduction: Traumatic brain injury (TBI) contributes to a substantial number of deaths and cases of permanent disability annually. Data from the CDC indicate a higher incidence and worse outcome of TBI in the aging population. The long-term disabilities suffered by survivors very often include cognitive deficits, which are attributable in part to the damage to the hippocampus. Previous work from our laboratory has indicated that higher expression of proinflammatory cytokines and chemokines in the aging brain might be responsible for higher vulnerability to injury. The higher activation of cytokines and chemokines in aging mice suggests increased activation of microglia and astrocytes.

Method: The study was designed to investigate the expression of markers of activated microglia and astrocytes in hippocampus of mice after injury to the sensorimotor cortex using a controlled cortical impactor in aged (21-24 mo) and adult (5-6 mo) mice using real-time RT-PCR and western blot analysis 1, 2, 3, 7, 14 and 28 days after injury.

Results: Higher basal expression of cd11b and IBA1, markers of activated microglia, was observed in aged hippocampus as compared to the adult mice. Expression increased gradually after injury and reached maximum after 3 days of injury in both groups. However, in the aged hippocampus the expression of microglial markers was higher at all time points and was prolonged. The expression was approximately 1.5 and 2 fold higher in aged than adult mice after 3 and 7 days of injury respectively. Expression of GFAP and S100B, markers of activated astrocytes, was higher in aged mice and reached maximum after 7 days of injury. The expression of astrocyte markers returned to near basal 28 days after injury in the adult mice, whereas in the aged mice levels were still elevated at 28 days. The immunohistochemical analysis of sections using IBA1 and GFAP antibodies also revealed higher glial activation in the aged brain after injury.

Discussion: The results from this study suggest that pronounced and prolonged activation of microglia and astrocytes in hippocampus may contribute to worse cognitive outcome in the elderly following TBI (Supported by AG026482)

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BEHAVIORAL OUTCOMES FOLLOWING BIOMECHANICALLY DISTINCT DIFFUSE BRAIN INJURIES

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Introduction: Input pulse magnitude and duration influence diffuse brain injury (DBI) severity. However, the role of duration was not clearly identified. This study's purpose was to determine effects of pulse magnitude and duration on behavioral outcomes following DBI. **Methods**: This protocol was approved by our Institutional Review Board. An experimental rat model induced mild DBI through coronal plane head angular acceleration. Experimental animals were divided into 3 groups: short duration/low angular acceleration (group 1), short duration/high angular acceleration (group 2), and long duration/low angular acceleration (group 3). Rats were anesthetized prior to and given reversal agent immediately following insult. Unconscious time was assessed through absence of corneal reflex and compared to controls to determine whether injury was sustained. After regaining complete consciousness, assessed by return of righting reflex, and within 1 hour of injury, all animals were placed in a y maze for 20 minutes and videographed from above. Videography was analyzed by a blinded observer and number of times animals changed y-maze arms quantified. This parameter was used as a measure of rat activity following injury. **Results**: Angular acceleration magnitudes were 427 ± 21 (low) and 502 ± 15 (high) krad/s/s and durations were 1.9 ± 0.2 (short) and 2.9 ± 0.1 (long) msec. 5, 6, and 5 animals were in groups 1, 2, and 3. 3 control animals underwent the entire protocol minus insult. Unconscious time (time from reversal agent administration to corneal reflex) was significantly longer than controls in all 3 groups. Number of y-maze arm changes (a measure of activity level) was significantly dependent upon experimental group. Post-hoc analysis revealed group 1 was not significantly different than controls. However, group 2 demonstrated a significantly decreased and group 3 demonstrated a significantly increased number of arm changes compared to controls and group 1. **Discussion**: Present results demonstrated that rats subjected to high magnitude pulses were less active while rats subjected to insults of longer duration were more active. This finding highlights a role of pulse duration and magnitude in DBI severity that may be indicative of different injuries.

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ROLE OF PROTEIN PHOSPHATASE 1 (PP1) IN CORTEX AND HIPPOCAMPUS AFTER LATERAL FLUID PERCUSSION (LFP) BRAIN INJURY IN RATS

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Introduction: Protein phosphorylation (kinases) and dephosphorylation (phosphatases) are widespread and dynamic biochemical processes that lead to many cellular changes including signal transduction. Previous studies in our lab have shown a rapid increase in levels of phosphorylated calcium/calmodulin-dependent kinase II (pCaMKII) within hippocampus and cortex at 10 and 30 min after LFP injury that was no longer elevated when examined at 3, 8, and 24 hrs. The subsequent dephosphorylation of pCaMKII may have been mediated by protein phosphatases. This particular study explored the role of a protein phosphatase, protein phosphatase 1 (PP1). Specifically, PP1 inactivates pCaMKII through deactivation of Inhibitor 1 (I1). PP1 is also implicated in such cellular processes as cell division, apoptosis, and long-term memory. PP1(γ1 subunit) is compartmentalized predominately in the cell soma but is also found in dendrites and presynaptic boutons where it co-localizes with CaMKII. **Methods:** Western immunoblotting was used to compare levels of PP1γ1 in both the ipsilateral and contralateral rat hippocampi and cortices of LFP injured and sham injured rats at 10 min, 30 min, and 3 hrs after injury. **Results:** Western blotting results showed there was no significant difference between ipsilateral and contralateral cortical and hippocampal regions at any time point after injury. Levels of PP1γ1 in the hippocampus were significantly higher in the TBI versus sham injured groups only at the 10 minute time point. In the cortex, there was no significant difference between TBI and sham at or between time points. **Discussion:** These results suggest that PP1γ1 levels are minimally and transiently altered after injury. These changes may not profoundly affect concomitant levels of pCaMKII and subsequent changes in cell injury and plasticity after brain trauma.

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EFFECT OF HEMORRHAGIC SHOCK ON NEURONAL DEATH AND CEREBRAL BLOOD FLOW AFTER EXPERIMENTAL TRAUMATIC BRAIN INJURY IN MICE: MAGNETIC RESONANCE IMAGING ASSESSMENT

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Introduction: Combined traumatic brain injury (TBI) and hemorrhagic shock (HS) worsen clinical outcomes vs TBI alone. Few studies have explored the effect of HS on neuronal death or cerebral blood flow (CBF) after TBI; none have been performed in mice. Perfusion Magnetic Resonance Imaging (MRI) allows serial assessments of the effect of HS and resuscitation after TBI, providing a unique opportunity to assess CBF in vulnerable brain regions.

Hypothesis: Hippocampal neuronal death in CA1 is exacerbated by HS after TBI and is dependent on the duration of HS and the reduction in CBF.

Methods: Male C57BL6J mice (n = 6/group), anesthetized with isoflurane, underwent mild-moderate controlled cortical impact (CCI) (5m/sec, 1.0 mm) followed by 60 or 90 min of volume-controlled HS, or continued anesthesia (CCI only). Naïve and HS only groups were also studied. In CCI + HS and HS only groups, mice were resuscitated with hetastarch for 30 min (pre-hospital) followed by return of shed blood for 60 min (hospital care). Neuronal damage in CA1 was assessed on d 7 (H&E) by a blinded evaluator. Also, preliminary studies in separate mice (n = 3/group) assessed CBF serially by perfusion MRI at 4.7T during HS (60 min) and during pre-hospital and hospital care phases.

Results: HS reduced MAP from a baseline of ~85 mmHg to ~35 mmHg during HS in HS only, CCI + 60 min HS and CCI + 90 min HS ($p < 0.05$ vs CCI only). MAP recovered to ~50 mmHg in pre-hospital and ~70 mmHg after hospital resuscitation. 90 min of HS dramatically exacerbated CA1 cell death vs CCI only or CCI + 60 min HS. CA1 counts in HS only, CCI only, CCI + 60 min HS and CCI + 90 min HS were 32.3 ± 7.6, 30.8 ± 6.8, 28.1 ± 2.2, and 16.5 ± 2.2, respectively, $p < 0.05$. There was no significant difference in CA1 between CCI only and CCI + 60 min HS. Hippocampal CBF was reduced similarly vs naïve at 60 min after CCI or CCI + HS. Studies of CBF at 90 min after TBI + HS are ongoing.

Conclusions: Surprisingly, 90 rather than 60 min of HS was required to significantly exacerbate CA1 neuronal death after mild-moderate CCI. This agrees with our CCI + 60 min HS MRI data, which did not show a further CBF reduction. Our findings suggest that although the traumatically injured brain has enhanced vulnerability to HS, a therapeutic window exists for benefit from optimized resuscitation after combined TBI + HS. Support: US Army PR054755 W81XWH-06-10247.

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DECAY IN QUALITY OF CHEST COMPRESSIONS WITH TWO FINGERS TECHNIQUE DURING LONE RESCUER INFANT MANIKIN CPR.

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Introduction: We previously reported that Two Thumb (TT) CPR generates higher compression depth (CD) and compression pressure (CP)compared to Two Finger (TF) in an infant manikin model. In an adult manikin study, chest compression depth (CD) and rate (CR) decreased after 1 minute of CPR secondary to fatigue. It is unknown if fatigue results in poor chest compressions during infant CPR. **Hypothesis:** We hypothesized that the TT technique produces better quality CPR, but may require more effort resulting in decay of chest compression quality compared to TF technique using the new compression:ventilation guideline. **Methods:** A Laerdal™ Baby ALS Trainer manikin was modified to digitally record CR, CD and CP. BLS or PALS-certified healthcare providers were randomized to perform 5 minutes CPR using 30:2 compression:ventilation ratio with either TT or TF technique. Subjects were blinded to data recording. The change in compression quality was analyzed by calculating the change over time (slope). Slopes (mean±SD) were analyzed using unmatched 2-sided t-test comparison of the slopes of CD and CP minute by minute, over 5 minutes. P-value ≤0.05 was considered significant. **Results:** Sixteen subjects were randomized to each group. The mean slopes over 5 minutes, between TF and TT technique showed significant deterioration of CD with TF technique, p <0.001. Although CP was significantly higher with TT technique, there was no significant reduction of CP over time in either group, p=0.81. CR was 133±34 per minute with TT vs. 136±37 with TF, p=0.81. **Conclusions:** Our study suggests that during lone rescuer infant CPR, TF technique is associated with a rapid decline in quality of CPR over 5 minutes. This effect was not seen with the use of Two Thumb technique, which suggests superior CPR performance especially over prolonged time. We recommend that the Two Thumb technique is preferred for infant CPR, especially when provided continuously in the infant with a secured airway.

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NITROXIDE-BASED RESUSCITATION OF COMBINED TRAUMATIC BRAIN INJURY AND HEMORRHAGIC SHOCK: EFFECT ON ACUTE HEMODYNAMICS.

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Introduction: Polynitroxyl albumin (PNA) is a novel colloid composed of albumin with 40 covalently linked nitroxide antioxidant moieties. It has protective effects in models of hemorrhagic shock (HS), stroke, and cerebral hemorrhage. Outcome after traumatic brain injury (TBI) is worsened by HS, but the optimal resuscitation approach remains unclear. We have developed a clinically relevant mouse model to study therapies for this combined insult. **Hypothesis:** Resuscitation with PNA is equal to or better than current civilian (lactated Ringers, LR) or military (Hextend, HX) standard of care fluids or hypertonic (3%) saline in TBI+HS. **Methods:** Isoflurane anesthetized C57BL6 mice (n=30) underwent controlled cortical impact to the left parietal cortex followed by 90 min of volume controlled HS (2cc/100g, MABP ~35-40 mmHg). After HS mice were randomized to resuscitation with LR, HX, 3%, or PNA. HS was followed by 30 min of fluid administration targeting MABP >50 mmHg (pre-hospital phase). Shed blood was then re-infused and a MABP of >70 mmHg targeted. MABP in each phase, amount of fluid given, arterial lactate, and 7d survival were assessed. **Results:** There was no difference between groups in MABP at the end of HS. However, pre-hospital MABP was higher in PNA and HX (both P<0.05 vs LR or 3%). Fluid requirements in the pre-hospital phase were less in PNA and HX (0.19±0.02 and 0.24 ± 0.07ml, respectively), both P<0.05 vs LR (0.95±0.44mL) or 3% (0.57±0.34mL). Improvement in lactate at end of resuscitation tended to be greater in PNA (-1.96±0.35) vs LR, HX, or 3% (-0.92±0.7, -0.81 ± 0.25, 0.10±0.85, respectively, P=0.132). 7d survival was 6/8 LR, 3/8 HX, 4/7 3% and 6/7 PNA, P=0.297. **Conclusions:** PNA exhibited favorable effects vs either LR or 3% in TBI+HS. Similar or favorable effects for PNA were seen vs HX. Studies are underway to assess the effect of these therapies on neuronal death, ICP, oxidative stress, and cognitive outcome.

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CAN THORACIC IMPEDANCE OBTAINED VIA DEFIBRILLATOR ELECTRODE PADS ACCURATELY DETECT RESCUE BREATHS IN CHILDREN?

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Introduction: Resuscitation guidelines recommend target values for rate and depth of rescue breathing. Thoracic impedance (TI) obtained via defibrillator electrode pads is FDA approved to detect and guide breaths in adults. Performance of this technology has not been studied in children. **Hypothesis:** TI obtained via Anterior-Anterior defibrillator pad location (AA) accurately detects guideline recommended 7-10 mL/kg breaths in children. Breath detection is superior with AA vs Anterior-Posterior pad location (AP). **Methods:** IRB approved, prospective pilot study of stable ICU patients (6 mos-17y) on conventional mechanical ventilation. Patients with chest tubes, obvious chest wall deformity, skin breakdown or inability to place pads in standard AA or AP location were excluded. TI was obtained via Philips MRx defibrillator with standard electrode pads (limit of detection >0.4 Ohms) for 5 min in AA and AP locations. Tidal volume(Vt) was simultaneously measured by pneumotach (Novametrix CO2SMO Plus). Analysis using descriptive statistics, chi-square, t-test, and Bland-Altman method, where appropriate. **Results:** 15 pts age 7.5(±5.5y) and weight 18.6kg (IQR 14.6-41) generated 13 AA and 13 AP evaluable episodes. In AA location, TI detected 825/858 breaths ≥7mL/kg (sensitivity 96.2%)and 360/489 breaths >2-7 mL/kg (sensitivity 73.6%). TI detected 87.9% (1185/1347) AA vs. 86.4% (1217/1409) AP location breaths >2 ml/kg (p=0.23). Limit of Vt detection per patient: 4.27 ± 1.5 ml/kg AA vs 4.86 ± 1.7 ml/kg AP (p = 0.36). Method comparison (AA vs. AP) of TI breath detection showed a mean bias of -1.8 mL/kg/Ohms (95% CI: -4.2, 0.7) with limits of agreement (precision) from -8.8 mL/kg/Ohms (95% CI: -13.1, -4.6) to 5.3 mL/kg/Ohms (95% CI: 1.0, 9.6). **Conclusions:** This pilot study demonstrates that thoracic impedance obtained via defibrillator pads accurately detects large (7-10 mL/kg) breaths in critically ill children. However, sensitivity to detect small (2-7mL/kg) breaths is limited. Breath detection is slightly superior with AA vs AP pad location. Support: Laerdal

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CPR FOR BRADYCARDIA/POOR PERFUSION IN CHILDREN: AN ANALYSIS OF THE NATIONAL REGISTRY OF CPR.

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Introduction: Bradycardia with pulse (BP) in children is an indication for chest compressions in some circumstances. Chest compressions (CC) for bradycardia with pulse (BP) in children has been associated in unadjusted analyses with improved survival outcomes compared with CC for pulseless cardiac arrest. **Hypothesis:** Patients who receive CC for BP before PEA/asystole develops have better survival to hospital discharge than patients receiving CC for PEA/asystole, even when adjusted for patient factors, pre-arrest clinical status, and process of resuscitative care. **Methods:** All patients < 18 years reported to the NRCPR database Jan 2002-Dec 2006 were eligible. Patients with bradycardia, asystole, or PEA and who received CC for > 1 minute were included. Patients newly born in the delivery suite, or with shockable first documented rhythm were excluded. Univariate analysis between patients receiving CPR for BP versus asystole/PEA was performed by chi-square for dichotomous variables and Wilcoxon rank sum for categorical variables. Variables analyzed inlcuded: survival to discharge, patient factors (age, event location, illness category), clinical factors (monitor status, respiratory support, cardiovascular support), and processes of care (invasive airway, epinephrine bolus, vasopressin bolus, duration of CC). Multivariate analysis was performed to assess CC for BP as an independent predictor of survival. **Results:** Complete data was available for 3149 patients. 1732 (55%) had BP and 640 (37%) survived to hospital discharge. Of 1417 (45%) with asystole/PEA, 331 (23%) survived to hospital discharge (p<0.001). After adjusting for confounding factors, CC delivery for BP was significantly associated with survival to hospital discharge (OR 1.57, 95% CI 1.28-1.93). **Conclusions:** Patients receiving CC for BP before pulselessness develops have better survival to hospital discharge, even when adjusted for patient factors, location, etiology of arrest, and process of resuscitative care.

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ASSOCIATION OF PROMOTER POLYMORPHISMS WITHIN ALPHA 7 NICOTINIC ACETYLCHOLINE RECEPTOR GENE WITH SEVERE SEPSIS.

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Introduction: Sepsis is a clinical syndrome characterized by systemic inflammatory reaction. Recent studies identified that vagal nerve served as an immunomodulator in inflammatory diseases. Stimulation of vagal nerve attenuates the production of proinflammatory cytokines, inhibits the inflammatory process and improves survival in experimental medels of sepsis. This anti-inflammatory pathway is mediated by the alpha 7 nicotinic acetylcholine receptor (CHRNA7), which indicates that CHRNA7 is a candidate gene in genetic study of sepsis. **Hypothesis:** This study is to investigate whether the promoter polymorphisms within CHRNA7 influence the clinical course of severe sepsis. **Methods:** Severe sepsis was diagnosed based on SCCM/ACCP criteria. The -1313A/G and -1512T/G variations were genotyped in 186 patients with severe sepsis and 326 matched healthy controls by means of PCR-RFLP. **Results:** The genotype frequency of -1313A/G in patients with severe sepsis was AA 40.9%, GA 43.5% and GG 15.6%, comparable to that of AA 38.1%, GA 43.4% and GG 18.5% in controls ($p>0.05$, Fisher's exact test). The genotype frequency of -1512T/G in patients with severe sepsis was TT 77.4%, GT 19.9% and GG 2.7%, similar to that of TT 74.5%, GT 24.3% and GG 1.2% in controls ($p>0.05$, Fisher's exact test). Meanwhile, there was no significant difference in the allelic frequency of these two SNPs in the defined groups. Furthermore, when the patients with severe sepsis were divided into survivors and non-survivors, neither -1313A/G nor -1512T/G contributed to the fatal outcome of severe sepsis. Logistic regression analysis also demonstrated that the two promoter polymorphisms were not independent factors for the susceptability to and fatal outcome of severe sepsis. **Conclusions:** These findings show that the promoter polymorphisms of -1313A/G and -1512T/G in CHRNA7 are not associated with severe sepsis, and may not serve as genetic markers for severe sepsis.

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CHRONIC COGNITIVE DEFICITS AND HEMISPHERIC ATROPHY ARE ASSOCIATED WITH EITHER CONTUSIVE OR NON-CONTUSIVE CLOSED HEAD INJURY IN THE IMMATURE RAT.

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Introduction: Brain trauma in infants and young children lead to long-term cognitive deficits that are associated with either focal contusions or diffuse lesions. Our purpose was to understand mechanisms underlying chronic cognitive deficits following focal or diffuse closed head injury in the immature rat. **Hypothesis:** Focal (contusive) closed head injury will lead to chronic cognitive deficits and a focal brain cavity, while diffuse (non-contusive) injury will lead to chronic cognitive deficits and diffuse brain atrophy. **Methods:** Anesthetized post-natal day 11 rats (neurologically a toddler) were subjected to an impact (5 m/sec, 3 mm depth) on the intact skull over the left parietal cortex (n= 4 injured/injury type/time point for histology; n= 10/injury type and 10 uninjured for behavioral analysis), with either a metal or a silicone-tipped indenter which produce contusive or non-contusive brain injury, respectively. **Results:** By 6 hr to 3 days following contusive injury, neurodegeneration and gliosis were observed in all layers of the ipsilateral cortex under the impact site along with neurodegeneration in the hippocampal dentate hilus and extensive traumatic axonal injury in the white matter and thalamus. A similar pathology was observed following non-contusive injury, except in the cortex where sporadic neurodegeneration was noted. By 3 to 7 days, neurodegeneration in the ipsilateral thalamus and axonal degeneration in the white matter were observed in all injured animals. At 2 weeks, injured animals exhibited diffuse cortical, white matter, and hippocampal atrophy in the injured hemisphere and was associated with learning and memory deficits ($p < 0.05$). In addition, impact with a metal tip also resulted in a small but overt focal lesion. **Conclusions:** Our results demonstrate that regardless of the mechanism of the initial impact, trauma to the immature brain resulted in chronic diffuse brain atrophy and cognitive deficits.

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EFFECT OF HEMORRHAGIC SHOCK ON THE MICROGLIAL RESPONSE AFTER TRAUMATIC BRAIN INJURY IN MICE.

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Introduction: Hypotension is a common secondary insult after traumatic brain injury (TBI) and is associated with poor outcome. We previously reported that hemorrhagic shock results a ~6-fold increase in hippocampal neuronal death in CA1 after controlled cortical impact (CCI) in a mouse model of TBI. HS may alter the inflammatory response to TBI by modulating microglial activation which could either exacerbate damage or enhance neuroprotection. **Hypothesis:** HS influences the microglial response to TBI. **Methods:** Isoflurane anesthetized C57BL6 mice were subjected to either HS (2cc/100g, MAP 35-40 mmHg) for 90 min, unilateral CCI, or combined injury (CCI+HS). After 90 min of HS, MAP was maintained >50 mmHg for 30 min with Hextend, followed by reinfusion of shed blood. Mice were killed at 7 days post-injury. Microglia were quantified (ImageJ software, NIH) in brain sections in ipsilateral and contralateral CA1 and CA3 hippocampus using FITC labeled anti-Iba-1 antibody. **Results:** HS alone produced no change in the number of microglia vs naïve controls. See Table. CCI increased the number of microglia bilaterally; ipsilateral > contralateral. The increase in ipsilateral microglia produced by CCI+HS was not significantly different from that produced by CCI alone. **Conclusions:** HS alone did not increase the number of microglia in mouse brain, while CCI results in a marked microglial response. After CCI, the microglial response was not augmented by HS, despite markedly greater neuronal death. Thus, a level of HS that augments neuronal death after TBI does not augment the microglial response. We speculate that HS may blunt an endogenous neuroprotective effect of microglial activation at 7d after TBI. Support: US Army PR054755W81XWH-06-10247 and NS30318 and NS38087

Table. Number of microglia per 200x field in CA1 and CA3

	Ipsilateral		Contralateral	
	CA1	CA3	CA1	CA3
HS (n=5)	18.7±2.1	15.3±2.3	13.6±2.0	12±2.2
CCI (n=5)	94.2±9.3*	92.7±13.2*	53.8±12.8*	40.5±2.5
CCI+HS (n=5)	78.5±13*	84.7±18.1*	42.8±10.4	31.8±11.5

*p<0.05 vs HS

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CEREBROSPINAL FLUID ATP LEVELS ARE ELEVATED IN PATIENTS WITH NONTRAUMATIC INTRACRANIAL HEMORRHAGE.

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Introduction: ATP and other nucleotides are released from many cell types into the extracellular milieu in response to stress. ATP stimulation of purinoceptors is important to cellular homeostasis and neurotransmission, although disregulation of purine signaling has been implicated in pathologic processes such as vasospasm and gliosis. ATP levels in the cerebrospinal fluid (CSF) of patients with acute intracranial pathology are poorly defined. **Hypothesis:** We hypothesized that ATP is elevated in the CSF of patients with nontraumatic intracranial hemorrhage. **Methods:** Seven patients had CSF samples obtained at the time of intraventricular drain placement for nontraumatic intracranial hemorrhage. Specific etiologies included intraventricular hemorrhage (n=1), intraparenchymal hemorrhage (n=2), and subarachnoid hemorrhage (n=4). Eight patients undergoing spinal anesthesia for elective surgical procedures provided CSF samples which served as controls. Samples were analyzed for ATP by luciferin-luciferase luminometry. Additionally, other markers of brain cellular injury (neuron specific enolase (NSE), S100B, and 8-Isoprostane) were measured by immunoassay. Student's t-test was used to compare means. Values are expressed as mean±standard error and two-tailed p values are reported. **Results:** CSF ATP levels were significantly elevated in patients with intracranial hemorrhage compared to controls (123±45 vs. 3±3 nM, $p=0.01$). Additionally, NSE and 8-Isoprostane levels were also significantly elevated (89±29 vs. 8±2 µg/L, $p=0.01$ and 34±8 vs. 10±9 pg/mL, $p=0.02$ respectively) Elevations in S100B did not reach statistical significance (110.6±78 vs. 0.5±0.2 ng/mL, $p=0.12$). No significant linear relationships between elevations in ATP and NSE, S100B, or 8-Isoprostane were observed. **Conclusions:** ATP is elevated to physiologic relevant levels in the CSF of patients who experience nontraumatic intracranial hemorrhage. These elevations in ATP parallel other markers of brain cellular injury. The role of ATP in aggravating or mitigating cell injury in this setting remains to be defined.

Reference 18. Proceedings from the NINDS Neurological Effects of Blast Injury Workshop.

Emergent Potential Treatments for Blast-Induced Neurotrauma

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Acute therapeutic approaches to blast-induced traumatic brain injury (TBI) target key aspects of a complex secondary injury cascade. Five factors must be considered, namely, 1) facets of TBI that are generally common across all forms of injury, 2) unique pathophysiological features of blast-induced TBI, 3) extracerebral insults (shock/polytrauma) that may complicate the injury, 4) severity of the insult, and 5) dose response and brain pharmacodynamics/kinetics. In addition, any acute therapeutic approach must account for critical temporal factors that influence the potential implementation of treatments in the field, emergency department, operating room, and ICU settings. Conventional facets of TBI that represent therapeutic targets include categories such as neuronal death, excitotoxicity, edema, axonal injury, oxidative stress, mitochondrial damage, ischemia, and inflammation, synaptic injury, and disturbances in cell signaling, among others. Although much remains to be discovered, preliminary data suggest that several aspects of TBI pathophysiology are of special importance in blast-induced neurotrauma—particularly in the setting of severe injury— including malignant edema, vasospasm, axonal injury, and intracerebral hemorrhage. It also must be recognized that any new therapies will be superimposed upon the current treatment regimen, which for severe blast-induced TBI is substantial, while for mild blast-induced TBI is limited. Two overarching approaches to therapy of blast-induced TBI will be discussed, namely, therapies that have been shown to have promise in conventional experimental models of TBI, or phase I-II clinical trials. These therapies represent "*low-hanging fruit,*"should be prioritized taking into consideration the aforementioned unique pathophysiological aspects of blast TBI, and tested in emerging experimental models of blast-induced TBI. In parallel, more speculative, but potentially higher yield targeted therapies should be explored, preferably, via high-throughput screening, in rodent models of conventional or blast TBI (across injury levels, gender, and with and without shock/polytrauma). And the most promising agents should be advanced from rodents to large animal models of blast-induced neurotrauma and clinical trials. Also, although there may be many shared mechanisms across injury levels in blast neurotrauma, optimal therapies for mild and severe blast-induced TBI are likely to differ. In addition to conventional approaches, a number of novel therapeutic approaches should also be explored including agents that target specific subcellular compartments (such as mitochondria), new delivery systems (such as nanoparticles targeting microglia/macrophages), acute application of cellular therapies, and new hemoglobin-based resuscitation fluids, among others. With the most promising therapies, some effort will be necessary to evaluate them in models that include various aspects of standard therapy. Given the complex nature of the secondary injury cascade in blast neurotrauma, combined therapies are also likely to be necessary to optimize outcome, particularly in the setting of severe injury. Finally, therapeutic strategies should set the stage for optimal rehabilitation/regeneration/re-wiring, in a continuum of care that goes from the field to rehabilitation. This overall approach to therapy development and selected specific examples of promising approaches in each category will be discussed.
Support: DARPA PREVENT program, CDMRP, NS38087, and NS30318

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CASE CONTROL STUDY ON DECOMPRESSIVE CRANIECTOMY IN CHILDREN WITH TRAUMATIC BRAIN INJURY

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Objectives: Decompressive craniectomy (DC) following traumatic brain injury (TBI) is performed to evacuate hematomas and to relieve intracranial pressure that may compromise viable brain tissue. The indications, type, and timing vary between centers, complications attributable to DC are reported to be rare but may have serious implications specifically in young children and may profoundly modify the length of care and recovery. We hypothesized that the odds of developing infectious complications in children would be different between children treated with DC for TBI compared to non-TBI indications.
Methods: We performed a case control study to characterize the complications and outcomes of children who required admission to the pediatric intensive care unit (PICU) and were treated with DC associated with TBI. Among 905 craniectomies registered in the surgical database of the Hospital for Sick Children between 2001 and 2006, we identified 333 subjects admitted to PICU among which 45 cases underwent DC associated with TBI. For every TBI case, three controls were selected and matched for age among children who underwent DC for urgent indications such as stroke, tumors, vascular malformations. Primary outcome was the overall incidence rate of complications; secondary outcomes were time to developing an infection, time to discontinuing mechanical ventilation, time to hospital discharge, and outcomes related to the primary disease such as hydrocephalus and functional outcome (PCPC & POPC).
Results: Among the 45 cases of TBI admitted to PICU treated with DC, 20 had severe TBI (Best GCS < 9), 42 survived (3 subjects who died had a best GCS of 3). Among survivors: one patient developed meningitis, while almost a third had other infections related to either mechanical ventilation or wound infections. All survivors were discharged with a GCS of 14 or 15 (82% had GCS 15), none were vegetative, nor required a tracheostomy. 95% of survivors had a PCPC/POPC of 3 or above i.e., favourable outcome on hospital discharge. Odds of developing infectious complications were not related to the indication of the DC but to the overall length of stay.
Conclusions. Complications associated with DC are rare and children who undergo decompressive craniectomy following TBI have favourable outcomes.

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SELECTIVE BRAIN COOLING ATTENUATES ELEVATED INTRACRANIAL PRESSURE INDUCED BY PENETRATING BALLISTIC-LIKE BRAIN INJURY IN RATS

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Elevation of intracranial pressure (ICP) following traumatic brain injury (TBI) is highly associated with poor prognosis. Whole body hypothermia has been considered clinically as a non-pharmacological strategy to reduce TBI-induced increase in ICP, but at the risk of introducing systemic side effects. In this study we achieved selective brain cooling (SBC) by extracranial cooling of the bilateral common carotid arteries (CCA) and examined its effects on the increase in ICP in a rat model of penetrating ballistic-like brain injury (PBBI).The time course of ICP after PBBI or sham surgery was monitored in isoflurane anaesthetized rats. In a separate group of rats, SBC started immediately after injury (i.e. within 1 min) lowered brain temperature ~3°C below normal levels within 30 min. Brain cooling was maintained for 2 h before spontaneous re-warming was allowed. The ICP and brain temperature of all animals were monitored continuously for the initial 3 h, and again for 5 min daily for 7 days. The results showed that immediately following PBBI the ICP increased steadily and peaked at 24 h post-injury to 4x the baseline level. Subsequently, ICP gradually recovered and returned to the normal by day 4 post-injury. However, in rats subjected to 2 h SBC the elevation in ICP was significantly attenuated. These results indicate that, in the absence of a craniotomy, acute, rapid, and selective induction of brain hypothermia has sustained beneficial effects on elevated ICP, which may provide a therapeutic substrate to facilitate neuroprotection and improve recovery from a PBBI.

Table. Effect of SBC on PBBI induced ICP Elevation in Rats (Mean ±SD)

Groups	ICP (mmHg) Baseline	5 min	3 h	24 h	48 h	72 h
Sham (n=11)	6.7 ± 1.9	6.3 ± 2.1	6.5 ± 3.3	9.5 ± 2.9	9.8 ± 2.3	10.6 ± 3.3
PBBI (n=12)	7.5 ± 1.6	104 ± 33**	19.4 ± 5.5**	37.2 ± 10.4**	30.4 ± 9.0**	23.6 ± 8.3**
PBBI+SBC (n=14)	7.7 ± 1.8	100 ± 38	12.8 ± 3.1*#	20.8 ± 6.7*#	20.6 ± 9.3*#	17.5 ± 6.0 #

** P<0.01 compared with sham group, # p<0.05, ## p<0.01 compared with PBBI group

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RESUSCITATION OF COMBINED TRAUMATIC BRAIN INJURY AND HEMORRHAGIC SHOCK WITH POLYNITROXYL ALBUMIN: EFFECT ON FLUID REQUIREMENTS, BLOOD PRESSURE, SURVIVAL AND NEUROPATHOLOGY

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Outcome after traumatic brain injury (TBI) is worsened by hemorrhagic shock (HS), but the optimal resuscitation approach remains unclear. The SAFE study (Finfer, 2004) identified higher mortality rates in TBI patients treated with albumin vs normal saline. The novel colloid polynitroxyl albumin (PNA) is composed of albumin with 60 covalently linked nitroxide antioxidant moieties, and is protective in models of HS, stroke, and cerebral hemorrhage. Currently used fluids include lactated Ringers (LR), the civilian standard, Hextend (HX) the military standard, and hypertonic (3%) saline. We tested the hypothesis that resuscitation with PNA is equal or superior to resuscitation with HX, LR or 3% after TBI+HS. Isoflurane anesthetized C57BL6 mice (n = 30) underwent controlled cortical impact followed by 90 min of HS (2.0ml/100g, [30% blood volume], MABP ~ 35mmHg). After HS mice were randomized to resuscitation with LR, HX, 3%, or PNA. HS was followed by 30 min of test fluid administration targeting MAP > 50 mmHg (pre-hospital phase). Shed blood was re-infused and MABP > 70 mmHg targeted (hospital phase). MABP in each phase, amount of fluid required, 7d survival, and hippocampal CA1 and CA3 neuron counts were assessed. There was no difference between groups in MABP at the end of HS. However, pre-hospital MABP was higher in PNA and HX (both $p < 0.05$ vs LR or 3%). Fluid requirements in the pre-hospital phase were less in PNA and HX (0.19 ± 0.02 and 0.24 ± 0.07mL, respectively, both $p < 0.05$ vs LR (0.95 ± 0.44mL) or 3% (0.57 ± 0.34mL). 7d survival was highest with PNA (6/7) vs 6/8LR, 3/8 HX, or 4/7 3%, but this was not significant $p = 0.297$. Ipsilateral hippocampal CA1 neuron loss (vs contralateral) did not differ between groups (LR 32 ± 18%, HX 37 ± 10%, 3% 30 ± 18%, and PNA 39 ± 14%, $p = 0.81$). Ipsilateral hippocampal CA3 neuron loss did not differ between groups (LR 23 ± 22%, HX 14 ± 18%, 3% 13 ± 30%, PNA 12 ± 25%, $p = 0.86$). PNA exhibited favorable effects on all acute resuscitation parameters vs either LR or 3% in TBI+HS, and was comparable to HX. Resuscitation with PNA did not influence hippocampal neuronal survival. Our data suggest that PNA confers acute benefit without deleterious effects after combined TBI+HS. Further studies are underway assessing the effect of these therapies on ICP, edema, oxidative stress, and cognitive outcome.
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P142

EFFECTS OF LIPID PEROXIDATION INHIBITION ON CALPAIN-MEDIATED CYTOSKELETAL DEGRADATION AFTER TBI

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Lipid peroxidation is a major form of oxidative damage that takes place early after traumatic brain injury (TBI). Data from our lab show that lipid peroxidation in cortical tissue peaks at 1 hr post controlled cortical impact (CCI)-TBI in CF1 mice and remains significantly increased over 12 hrs after injury (Deng, et al. Exp Neurol. 2007 May;205(1):154-65). Lipid peroxidation is suggested to contribute to post-TBI calpain-mediated cytoskeletal (α-spectrin, 280 kDa) degradation that culminates in neuronal damage and neurological deficit after TBI. Detection of spectrin breakdown products, the 145-kDa fragment in particular, is used to estimate calpain-mediated cytoskeletal degradation since it provides a convenient biomarker of post traumatic calpain activation (Pineda, et al. J Neurotrauma. 2004 Oct;21(10):1443-56).The aim of this study was to further define the role of lipid peroxidation after TBI using the potent lipid peroxidation inhibitor U-83836E (Hall, et al. J Pharmacol Exp Ther. 1991 Aug;258(2):688-94). The study investigated the ability of U-83836E to ameliorate calpain-mediated cytoskeletal degradation. Male CF1 mice were randomized into sham, saline treated and U-83836E treated (0.1, 0.3, 1.0, 3.0, 10.0, and, 30.0 mg/kg) groups. The sham group received only craniotomy with no further treatment, whereas both saline- and U-83836E-treated groups received craniotomy and were subjected to severe (1.0 mm impact depth) CCI-TBI followed by I.V (tail vein) injection of the assigned treatment 15 minutes post injury. The levels of calpain-mediated cytoskeletal degradation products were significantly increased in injured cortical tissue at 24 hrs post-injury (950% over sham). U-83836E produced a dose-related attenuation of spectrin degradation compared to vehicle treatment. The best dose (3.0 mg/kg) reduced the levels of the calpain specific 145-kDa fragment by 70% ($p < 0.05$). The findings indicate that lipid peroxidation is an early event that contribute to post-TBI calpain activation. Currently we are using the best dose (3.0 mg/kg) to determine U-83836E therapeutic time window for decreasing calpain-mediated cytoskeletal degradation after CCI-TBI.
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Reference 20. Proceedings from the 26th Annual National Neurotrauma Symposium

Pathophysiology guided therapeutic approaches to blast-TBI and polytrauma

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Acute therapeutic approaches to blast-induced traumatic brain injury (TBI) target key aspects of a complex secondary injury cascade. Five factors must be considered, namely, 1) facets of TBI that are generally common across all forms of injury, 2) unique pathophysiological features of blast-induced TBI, 3) extracerebral insults (shock/polytrauma) that may complicate the injury, 4) severity of the insult, and 5) dose response and brain pharmacodynamics/kinetics. In addition, any acute therapeutic approach must account for critical temporal factors that influence the potential implementation of treatments in the field, emergency department, operating room, and ICU settings. Conventional facets of TBI that represent therapeutic targets include categories such as neuronal death, excitotoxicity, edema, axonal injury, oxidative stress, mitochondrial damage, ischemia, and inflammation, synaptic injury, and disturbances in cell signaling, among others. Although much remains to be discovered, preliminary data suggest that several aspects of TBI pathophysiology are of special importance in blast-induced neurotrauma—particularly in the setting of severe injury— including malignant edema, vasospasm, axonal injury, and intracerebral hemorrhage. It also must be recognized that any new therapies will be superimposed upon the current treatment regimen, which for severe blast-induced TBI is substantial, while for mild blast-induced TBI is limited. Two overarching approaches to therapy of blast-induced TBI will be discussed, namely, therapies that have been shown to have promise in conventional experimental models of TBI, or phase I-II clinical trials. These therapies represent "*low-hanging fruit,*"should be prioritized taking into consideration the aforementioned unique pathophysiological aspects of blast TBI, and tested in emerging experimental models of blast-induced TBI. In parallel, more speculative, but potentially higher yield targeted therapies should be explored, preferably, via high-throughput screening, in rodent models of conventional or blast TBI (across injury levels, gender, and with and without shock/polytrauma). And the most promising agents should be advanced from rodents to large animal models of blast-induced neurotrauma and clinical trials. Also, although there may be many shared mechanisms across injury levels in blast neurotrauma, optimal therapies for mild and severe blast-induced TBI are likely to differ. In addition to conventional approaches, a number of novel therapeutic approaches should also be explored including agents that target specific subcellular compartments (such as mitochondria), new delivery systems (such as nanoparticles targeting microglia/macrophages), acute application of cellular therapies, and new hemoglobin-based resuscitation fluids, among others. With the most promising therapies, some effort will be necessary to evaluate them in models that include various aspects of standard therapy. Given the complex nature of the secondary injury cascade in blast neurotrauma, combined therapies are also likely to be necessary to optimize outcome, particularly in the setting of severe injury. Finally, therapeutic strategies should set the stage for optimal rehabilitation/regeneration/re-wiring, in a continuum of care that goes from the field to rehabilitation. This overall approach to therapy development and selected specific examples of promising approaches in each category will be discussed.
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P57

SYSTEMATIC REVIEW ON DECOMPRESSIVE CRANIECTOMY IN CHILDREN FOLLOWING SEVERE TRAUMATIC BRAIN INJURY

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Objective: Decompressive Craniectomy (DC) remains a controversial therapy in children with severe Traumatic Brain Injury (TBI). We undertook a systematic review to assess the quality and quantity of the evidence and highlight the gaps found in the literature on Decompressive Craniectomy performed in children.
Methods: Medline, The Cochrane Library, EMBASE and OVID Medline were searched (June 1950 - July 2017). Studies were selected using a priori inclusion criteria using the following search terms (individually or combined): (decompressive) craniectomy, children, treatment and craniotomy. Two reviewers independently selected the articles, search terms; three reviewers read and extracted the content to achieve a minimally biased review. Given the paucity of controlled trials, we included both observational and experimental studies.
Results: Among 1778 studies retrieved, 31 met inclusion criteria. 83% were observational. Only one study used a randomized controlled trial design. Twenty eight articles (90%) had a study sample of less than 60 subjects, and the average study sample size (out a total of 837 subjects) per study was 27 subjects. Seventeen of the 31 articles exclusively studied a pediatric sample; only 20% of all the subjects studied were younger than 19 years. Uncontrollable intracranial pressure was the main indication for Decompressive Craniectomy in 20 studies. Glasgow Outcome Scale was used as functional endpoint in more 70% of the studies. Craniectomy lead to an improvement in intracranial pressure in more than half of subjects (53%), combined with a 70% better functional and neurological outcome status. Over 20 studies' conclusion statements, support Decompressive Craniectomy as a secondary therapy in children with TBI.
Conclusion: Due to the limited number of studies available and the studies' designs, we plan to perform a case-control study in children to examine the effects of the timing of Decompressive Craniectomy and co-interventions (e.g., antibiotic use), on the rate of complication, co-morbidities and outcomes. We look forward to the upcoming completion of the two randomized controlled trials in adults.

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EFFECT OF TAURINE WITH MAGNESIUM SULFATE ON RESPIRATORY CHAIN ENZYMES OF MITOCHONDRION IN RATS WITH ACUTE SEVERE TRAUMATIC BRAIN INJURY

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Object To investigate the activity of respiration chain enzymes of mitochondrion and the effects of taurine (Tau) with magnesium sulfate ($MgSO_4$) on the rats with acute severe traumatic brain injury. **Method** Forty male Sprague-Dawley rats (weight 300 ± 10g) were randomly divided into five groups: sham group, TBI group, Tau group, Tau-$MgSO_4$ group and $MgSO_4$ group. All animals were subjected to severe left brain injury by lateral fluid percussion device (1.7-2.0atm) except sham group. Tau (200 mg/kg), $MgSO_4$ (96 mg/kg) and Tau with $MgSO_4$ (Tau 200 mg/kg, Tau: Mg^{2+} = 2:1) were immediately injected by tail vein of rats after TBI for 7 days. Then both of the left and right brain samples were dissected and homogenized. Mitochondrion was extracted by density and speed-centrifugation. The activity and purity of mitochondrion were identified by Janus green B staining and electron microscopy (EM) respectively. Protein of mitochondrion was quantified according to Bradford. The activity of mitochondrial respiration chain enzymes which include succinate dehydrogenase(SDH), cytochrome c oxidase (CCO), reduced nicotinamide adenine dinucleotide(NADH) was measured by ultraviolet radiation (UV) spectrophotometer. All data was analyzed by one-way ANOVA. **Result:** The activity of mitochondrion was approved well by Janus green B dye. And the ultrastructure showed that there is more than 90% mitochondrion in an eyeshot, which substantiated the purity of mitochondrion. The activity of SDH (U/mg) of injured brain (left brain) in TBI group, Tau group, Tau-$MgSO_4$ group and $MgSO_4$ group, which were 0.90 ± 0.25, 1.38 ± 0.52, 2.08 ± 1.47, 0.60 ± 0.35 respectively, decreased significantly compared with sham group (3.19 ± 1.50). Only the SDH activity of Tau-$MgSO_4$ group was significantly increased than that of TBI group. For right brain, the SDH activity of Tau-$MgSO_4$ group significantly increased (5.25 ± 1.86) and of other groups (2.87 ± 1.73, 2.45 ± 1.81, 0.45 ± 0.24 in TBI, Tau, $MgSO_4$ group respectively) was decreased significantly compared with sham group (4.66 ± 2.36). For left and right brain, the activity of CCO (μmol/mg) is no statistic difference among 5 groups (0.59 ± 0.24, 0.53 ± 0.22, 0.46 ± 0.28, 0.52 ± 0.28, 0.49 ± 0.32 in left brain and 0.23 ± 0.06, 0.36 ± 0.20, 0.27 ± 0.24, 0.17 ± 0.10, 0.39 ± 0.22 in the right brain for sham group, TBI group, Tau group, Tau-$MgSO_4$ group and $MgSO_4$ group respectively). The CCO activity of right brain was decreased markedly than that of left brain in 4 groups except Tau group. The activity of NADH (U/mg) of injured brain (left brain) in TBI group (28.74 ± 7.99) and Tau-$MgSO_4$ group (23.02 ± 9.62) was decreased markedly than that of Tau group (40.10 ± 7.70), $MgSO_4$ group (37.74 ± 2.24) and sham group (39.49 ± 5.02). There was no difference in the right brain among 5 groups (39.06 ± 4.54, 27.16 ± 13.12, 31.66 ± 5.27, 39.12 ± 11.76, 37.13 ± 9.55 in the right brain for sham group, TBI group, Tau group, Tau-$MgSO_4$ group and $MgSO_4$ group respectively). **Conclusion** Mitochondrial respiration chain is the basic source that is quite important for energy supply. Our study demonstrates that the activity of SDH, NADH was significantly decreased and CCO activity can be normal after TBI. Taurine can improve markedly the activity of SDH, NADH at 7 days from the vein of rats, much better than the effect of $MgSO_4$ and Tau-$MgSO_4$ that can only increase the activity of NADH or SDH respectively. It means that taurine is a better agent to protect the function of mitochondrion after TBI.

Keywords Traumatic brain injury, Taurine, magnesium sulfate, mitochondrial respiration chain enzymes

P59

POLYNITROXYLATED PEGYLATED HEMOGLOBIN SOLUTION FOR THE ACUTE LIMITED FLUID RESUSCITATION OF HEMORRHAGIC SHOCK AFTER TRAUMATIC BRAIN INJURY IN A MOUSE MODEL

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In austere environments, such as after blast-induced traumatic brain injury (TBI) in Operation Iraqi Freedom, early resuscitation of hypotension to prevent secondary damage after TBI can be challenging. Though isotonic crystalloid or colloid solutions are typically used for this purpose, novel hemoglobin (Hb) based resuscitation fluids may have advantages in volume requirements, restoration of hemodynamics, and oxygen delivery. However, concerns with possible nitric oxide (NO) consumption and/or oxidative stress from free Hb have been raised (Natanson et al., 2008). The novel nitroxide antioxidant Hb solution, polynitroxylated pegylated Hb (PNPH), may confer an advantageous profile in this regard. We hypothesized that acute volume resuscitation with a 4% PNPH solution would be favorable compared to Lactated Ringer's solution (LR) or Hextend (the current civilian and military standards of care) in a mouse combined injury model. Isoflurane anesthetized C57BL6 mice were subjected to controlled cortical impact (5 m/s, 1 mm depth) followed by hemorrhage (2cc/100g, 1~30% blood volume). MAP 35-40 mmHg) for 90 min. After 90 min, to simulate limited prehospital resuscitation, MAP was maintained > 50 mmHg for 30 min with PNPH, LR, or Hextend. After 30 min, shed blood was re-infused. Blood pressures were recorded every 5 min during resuscitation. Resuscitation volumes were recorded at the end of the pre-hospital phase. Arterial blood gasses and glucose levels were monitored. Mortality rate did not significantly differ between groups, although mortality was highest with LR (1/6 PNPH vs 4/9 LR vs 0/5 Hextend). Resuscitation with PNPH (0.18 ± 0.05 ml) or Hextend (0.26 ± 0.07 ml) required less volume than with LR (0.96 ± 0.28 ml) ($p < 0.05$). PNPH (64.4 ± 2.9 mmHg) but not Hextend (58.8 ± 2.9) exhibited a significantly higher pre-hospital mean MAP vs LR (50.4 ± 2.9, $p < 0.05$) during resuscitation. Similarly, PNPH but not Hextend exhibited a significantly higher pre-hospital peak MAP vs LR ($p < 0.05$). Our results suggest that limited resuscitation with PNPH in the setting of combined TBI + hemorrhagic shock may offer advantages over LR or Hextend. Further investigation of neuropathology, tissue O_2 levels and markers of NO and oxidative stress are ongoing to determine if PNPH offers additional therapeutic advantages.

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BRAIN ENERGY DEPLETION CAUSED BY A DIFFUSE HEAD INJURY IN RATS IS NOT AMELIORATED BY THE INFUSION OF SODIUM LACTATE

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Traumatic brain injury (TBI) causes a substantial energy demand on cerebral metabolism to restore the biochemical and functional brain homeostasis. Recent findings suggest that lactate could be used by the brain as an alternative substrate to glucose in situations of impaired energy metabolism. This study was carried out to investigate the effects on cerebral metabolism of sodium lactate (NaLac) solutions infused to rats undergoing a severe diffuse TBI. After TBI rats were randomly assigned to one of the following treatment groups (n = 10 per group): TBI-normal saline, TBI-hypertonic saline, TBI-100mM NaLac, TBI-500mM NaLac, TBI-1280mM NaLac, TBI-2000mM NaLac and TBI-500mM NaLac plus magnesium sulphate. In addition, ten rats without trauma treated with normal saline were used as sham animals. Whole cerebrums were removed 6 hours post-injury and HPLC analysis of cerebral extracts were performed in order to measure those metabolites representative of the cell energy state, adenosine tri-phosphate (ATP) and ATP-catabolites; as well as nicotinic coenzymes (NAD+, NADH), N-acetylaspartate (NAA), tissue antioxidant defenses (ascorbic acid, glutathione) and molecules indicative of oxidative/nitrosative cellular damage (malondialdehyde and ADP-ribose). Following TBI, a significant reduction in the cerebral content of ATP, NAD+, NAA, ascorbic acid and glutathione was observed, while the brain levels of ATP-catabolites, malondialdehyde and ADP-ribose were elevated. These metabolic alterations did not changed significantly in any of the groups treated with NaLac solutions. The metabolic pathway necessary to consume lactate may not be functional after a severe TBI due to a cerebral depletion of NAD+, an essential co-enzyme for the activity of the mitochondrial lactate-dehydrogenase.

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CAUSE FOR A PAUSE DURING CPR...IT MATTERS!

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Introduction: Improved CPR quality is associated with better survival outcomes, yet substandard CPR quality is common. Pauses in chest compressions (CC) are associated with worse survival. A first step to improve CPR quality is identifying factors that lead to substandard CPR. **Hypothesis:** Substandard CPR quality will be more likely to occur immediately after a change in CC provider compared to other causes for pauses in CC. **Methods:** With IRB approval, CPR quality was assessed during consecutive in-hospital CC events in children ≥8yrs with force transducer/accelerometer recordings. Data included: CC depth, rate, leaning force, and CC pause duration. Real-time computer audio feedback was supplied when rate was <90 or >110 CC/min, depth <38mm, leaning force >2.5kg, and pause >15s. Causes for pauses were identified by post-event debriefing (CPR team interviews) plus two independent reviews of stored CC data. Continuous CPR quality variables analyzed with descriptive summaries/Student t-test. RR of CPR error after a pause calculated. **Results:** We analyzed 190min of CPR and 117 pauses (median: 6.2s, IQR 3.2-14s) from 14 consecutive cardiac arrests. Causes for pauses were 52% CC provider switch (median 4s, IQR 2.8-8.7s), 23% pulse/rhythm analysis (median 9.8s, IQR 7.3-16.5s), 8% defibrillation attempt (median 22.4s, IQR 13.9s-34.4s), and 17% "other/undetermined" (median 4.4s, IQR 1.9-13.8s). During the first 5s of CC following pauses, leaning was more likely after CC provider switch (RR=3.1, 95% CI=1.8-5.2, p<0.0001) and to exceed the feedback trigger (≥ 5 consecutive CC with leaning >2.5kg) than for all other causes for pauses combined (RR=3.8, 95% CI=1.5–9.1, p<0.002). Provider switch resulted in shallower CCs (44±7 vs. 46±7mm, p=0.04) in the first 5s after switch. **Conclusions:** During in-hospital pediatric CPR, the majority of pauses are for provider switch. Substandard CPR quality (leaning/shallower CC) is more likely immediately after CC provider switch compared to other causes for CC pauses. Future studies should investigate the effect of feedback targeted to this high risk switch period.

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POLYNITROXYLATED PEGYLATED HEMOGLOBIN FOR THE ACUTE LIMITED FLUID RESUSCITATION OF HEMORRHAGIC SHOCK AFTER TRAUMATIC BRAIN INJURY IN MICE.

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Introduction: Resuscitation of hemorrhagic shock (HS) to prevent secondary injury after traumatic brain injury (TBI) is challenging. Though isotonic solutions are typically used, novel hemoglobin (Hb) based fluids may have advantages in volume requirement, restoration of hemodynamics, and oxygen delivery. However, concerns with NO consumption and oxidative stress from free Hb have been raised (Natanson et al., 2008). The novel second generation nitroxide antioxidant Hb, polynitroxylated pegylated Hb (PNPH), may be advantageous in this regard. **Hypothesis:** Acute resuscitation with a 4% PNPH solution will be favorable compared to Lactated Ringer's solution (LR) or Hextend (Hex). **Methods:** Isoflurane anesthetized C57BL6 mice (n=20) were subjected to controlled cortical impact (CCI) (5 m/s, 1 mm depth) followed by HS (2cc/100g, [~30% blood volume], MAP 35-40 mmHg) for 90 min. MAP was then maintained >50 mmHg for 30 min with PNPH, LR, or Hex. After 30 min, shed blood was infused. Blood pressures during resuscitation and fluid volumes to achieve pre-hospital MAP were recorded. Neurodegeneration at 7 days was examined using Fluoro-Jade C (FJC) staining. **Results:** Resuscitation with PNPH (0.18±0.05 ml) or Hex (0.26±0.07 ml) required less volume than LR (0.96±0.28 ml) (p<0.05). PNPH (64.4±2.9 mmHg) but not Hex (58.8±2.9) produced higher pre-hospital mean MAP vs LR (50.4±2.9, p<0.05). Similarly, PNPH but not Hex exhibited a higher pre-hospital peak MAP vs LR (p<0.05). Mortality did not differ significantly between groups. Mice resuscitated with PNPH had fewer FJC+ neurons in vulnerable regions of the hippocampus (p<0.05). **Conclusions:** Limited resuscitation of combined CCI + HS with PNPH offers advantages over LR or Hex including reduced volume requirements, better normalization of hemodynamics, and protection against neuronal death at 7 days. Support: US Army PR054755 W81 XWH-06-10247

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FEASIBILITY OF HEMODYNAMIC ASSESSMENT OF CPR QUALITY DURING IN-HOSPITAL ADOLESCENT ARREST.

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Introduction: AHA guidelines recommend CC depth of ≥33% anterior-posterior (AP) chest depth for children. During in-hospital pediatric resuscitation, direct association of CPR quality with improved blood pressures (BPs) has not been demonstrated. **Hypothesis:** CC that meet the target depth of ≥33% AP chest depth will result in higher BPs, compared to shallower CC, during real in-hospital CPR. **Methods:** With IRB approval, CPR quality was assessed with force transducers/accelerometers during consecutive in-hospital CC events in children ≥8yrs with peripheral arterial catheters. Data included: CC depth, CC force, and arterial BP. Simultaneous arterial BP associated with the force and depth of each CC was determined. Continuous CPR quality variables/BP analyzed with descriptive summaries. BP generated by CC ≥33% AP chest depth compared to shallower CC by Student t-test. **Results:** Analysis yielded 1481 evaluable CCs during 4 arrests (age range 14.5-16.9 yrs, chest depth 140-210mm). BPs were higher when CC depth was ≥33% AP chest depth: systolic 87±25 vs. 69±16 (p<0.0001); mean 52±13 vs. 43±9 (p<0.0001); diastolic 34±8 vs. 30±6 (p<0.0001). Figure 1: systolic BP vs. percent AP chest depth. **Conclusions:** Association of real-time in-hospital CPR data to hemodynamic effect is feasible. CC that achieved AHA recommended target depth of ≥33% AP chest depth resulted in higher systolic, mean and diastolic BPs, compared to shallower CC. In the future, appropriate control for quality of CPR and associated hemodynamics is important.

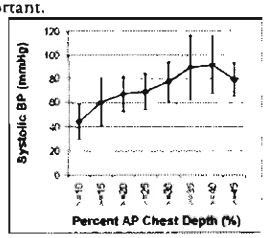

Figure 1: Systolic BP vs. Percent AP Chest Depth

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NON INVASIVE CONTINUOUS POSITIVE AIRWAY PRESSURE AND HIGH DOSE INTRAVENOUS NITROGLYCERIN IN HYPERTENSIVE PULMONARY EDEMA; WHICH ONE HAS AN OUTCOME IMPACT?

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Introduction: Non invasive continuous positive airway pressure (CPAP) has established superiority over conventional methods of management including low dose intravenous Nitroglycerin (IVNG) in patients presenting with hypertensive pulmonary edema (HPE). **Hypothesis:** Evaluation of the outcome of the use of high-dose intravenous nitroglycerin (HDNTG) versus non invasive CPAP in HPE. **Methods:** 60 patients with HPE were randomly assigned into 3 management groups; Conventional oxygen therapy (high flow oxygen through a non re-breathing face mask at 15 Liters/minute), noninvasive CPAP (10 cm.H2O via an oronasal mask) or HDNTG (IV boluses of 2 mg of NG every 5 minutes according to the response up to10 doses. Furosemide, conventional doses of nitrates and morphine had been given according to the clinical condition of the patients. **Results:** There was no statistically significant difference between the three groups regarding baseline characteristics. Predefined criteria of success were met in 30%, 50% and 90% of patients in the Oxygen, CPAP and HDNTG groups respectively p=0.009. The need for Mechanical Ventilation (MV) was required during the first two hours of treatment in 65%, 50% and 0% in the Oxygen, CPAP and HDNTG groups respectively p <0.001. The mean duration of ICU stay was 6.90±1.97, 5.60±1.67 and 4.00±1.84 days in the Oxygen, CPAP and HDNTG groups respectively p<0.001. Hypotension developed only in one patient in HDNTG group. The combined end point (death, need for MV and incidence of MI within 24 hours of admission) was met in 70%, 50% and 10% in the oxygen, CPAP and HDNTG groups respectively p=0.007. **Conclusions:** Not only it caused a prompt clinical improvement, HDNTG was superior to CPAP in reducing the need for MV, incidence of MI and duration of ICU stay. It was superior to CPAP in improving the combined end point of myocardial infarction within 24 hours of admission, need for mechanical ventilation and death.

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A PROSPECTIVE RANDOMIZED CROSSOVER TRIAL OF PEDIATRIC SIMULATION...DON'T JUST TALK ABOUT IT, JUST DO IT!

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Introduction: The value of repetitive simulation of specific scenarios compared to repetitive didactic training only for specific scenarios has not been well studied. **Hypothesis:** Our objective was to determine if case specific simulation training (ST) improves time to treatment for critical events. **Methods:** This prospective study randomized 25 pediatric residents to one of two groups: Pulseless ventricular tachycardia-torsades de pointes (pVT-T) (Group 1) or supraventricular tachycardia-poor perfusion (ppSVT), (Group 2). Scenarios done with Simbaby (Laerdal, Denmark) at three times: Initial (T0), 2 months (T2), and 4 months (T4). At all times both groups were given standardized didactics on all PALS algorithms, including pVT-T. At T0 and T2, Group 1 had ST for VF and pVT-T, Group 2 had ST for VF and ppSVT. At T4 Groups 1 and 2 had ST for VF, pVT-T, and ppSVT. Prior to T4, Group 1 had no ST for SVT and Group 2 had no ST for pVT-T, they only had didactics for management of these specific events. Primary outcomes were time to defibrillation (Defib) and Mg dose for pVT-T, and cardioversion and adenosine for ppSVT. Secondary outcomes were time to initiation of BVM, and duration of interruption of chest compressions from T0 to T4. Analysis by related and unrelated sample Wilcoxon tests. **Results:** At T4, Group 1 (n=14) had shorter time to shock (68s vs 108s, p=0.07) and Mg dose (37.5s vs 171s, p=0.002) compared to Group 2 (n=11). Group 2 had slightly shorter time to shock (66s vs 106s, p=0.119), and time to adenosine dose (35s vs 144s, p=0.09) that approached significance compared to Group 1. Time to BVM was reduced from T0 to T4 in VF in both Groups (n=25), 17.5s vs 8s, (p=0.011), as was time of interruptions during chest compressions (24s vs 15s, p=0.019). **Conclusions:** Repetitive case specific simulation training improves time to treatment for critical events compared to when repetitive didactic training only is performed for that specific scenario.

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SURVIVAL ANALYSIS AFTER CARDIOPULMONARY RESUSCITATION IN RATS WITH MYOCARDIAL INFARCTION TREATED WITH MESENCHYMAL STEM CELLS.

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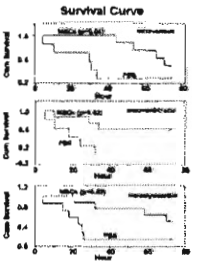
Introduction: Allogeneic bone marrow mesenchymal stem cells (MSCs) were administrated in a rat model of myocardial infarction by three different routes of administration including intravenous, intraventricular and intramyocardial injection. **Hypothesis:** Survival after cardiopulmonary resuscitation (CPR) would be improved in myocardial infarction animals treated with MSCs. **Methods:** Myocardial infarction was induced by ligation of the left anterior descending coronary artery in 54 rats (6 groups, 9 rats for each group). One month later, animals were randomized to receive 5×10^6 MSCs labeled with PKH26 or PBS into right femoral vein or the left ventricular cavity or the infarction zone in the anterior ventricular free wall. 4 weeks after MSCs or PBS injection, 6 min of ventricular fibrillation (VF) and 6 min of CPR were performed prior to defibrillation. Survival after resuscitation was recorded **Results:** There were no difference in success rate of resuscitation, number of surviving to 72 hours and number of shocks at paired groups. However, survival were siginificantly increased after CPR in MSCs treated groups comparing with the corresponding PBS treated groups (Figure 1). **Conclusions:** Survival after CPR were comparably improved in all groups of animal treated with MSCs in constrast to the PBS groups.



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RESUSCITATION OF TRAUMATIC BRAIN INJURY AND HEMORRHAGIC SHOCK WITH NOVEL COLLOIDS: EFFECTS ON ACUTE HEMODYNAMICS AND SURVIVAL.

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Introduction: Traumatic brain injury (TBI) with hemorrhagic shock (HS) contributes to civilian and military mortality, but the ideal resuscitation fluid is unknown. The SAFE study suggested colloids vs crystalloid confer deleterious effects after TBI (Myburgh, 2007). This is significant as the colloid Hextend (HEX) is used for combat resuscitation, and novel colloids, such as polyitroxyl albumin (PNA), have been created. PNA, albumin covalently linked to nitroxyl moieties, used alone and with free nitroxide Tempol (PNA+), had favorable effects in HS, stroke, and cerebral hemorrhage models (Kentner, 2002). **Hypothesis:** Resuscitation with colloids PNA, PNA+, albumin (ALB), or HEX confer favorable hemodynamic effects without worsening survival vs Lactated Ringers (LR) in our model. **Methods:** Isoflurane anesthetized C57BL6 mice (n=40) underwent controlled cortical impact and 90min of HS (20ml/kg, MAP 35mmHg). Mice were randomized to resuscitation with LR, HEX, ALB, PNA or PNA+, then entered 30min prehospital phase with fluid administration targeting MAP>50mmHg. Shed blood was returned and MAP>60mmHg targeted (inhospital phase). MAP, volume infused, and 7d survival were assessed. **Results:** MAP at end of HS did not differ between groups. PNA, PNA+, HEX, ALB reached higher MAP in prehospital phase vs LR ($p<0.01$). Prehospital and total fluid needs were less for PNA, PNA+, HEX, ALB vs LR ($p<0.01$). No group required more than shed blood during inhospital phase. Although not significant, PNA+ had the best numeric 7d survival (7/8 vs 5/8LR, 6/8HEX, 5/8PNA, 6/8ALB). **Conclusions:** In our model, colloids PNA, PNA+, HEX and ALB provided positive effects on hemodynamics with less volume vs LR without worsening survival. We are now examining neuopathology to determine if colloids mediate neuroprotection. Our findings corroborate work by Baker, 2008, and suggest dismissal of colloid resuscitation in TBI+HS may be premature.

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BRAIN CELLULAR RESPONSE AND TEMPERATURE MANIPULATION AFTER CARDIAC ARREST RESUSCITATION IN RATS.

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Introduction: Therapeutic hypothermia after cardiac arrest (CA) improves survival and functional outcomes whereas hyperthermia is harmful, but its neuroprotective mechanism remains unclear. We previously showed that hypothermia post-CA led to better neuro-electrical and behavioral recovery. **Hypothesis:** We quantified the impact of temperature manipulation on brain injury with stereological technique on microglia (by ionized calcium binding adaptor molecule 1 - Iba1), astrocytes (by glial fibrillary astrocyte protein - GFAP), and neurons (by Cresyl-violet staining). **Methods:** Based on 6 hours of immediate post-CA hypothermia (T=33°C), normothermia (T=37°C), or hyperthermia (T=39°C), 24 rats were evenly divided into 3 groups. Temperature was maintained using surface cooling and warming. Neurological recovery was evaluated using the Neurological Deficit Score (NDS). Histological evaluation was done after 72 hrs post CA. **Results:** Better recovery by NDS 72 hours post-CA was noted in rats treated with hypothermia (median, interquartile range: 74, 61-74), compared to normothermia (49, 47-61), and hyperthermia (43, 0-50) ($p<0.001$). There was a significantly different proportion of ischemic neurons by HDS in cortex (hypothermia: 19.7±3.4%, normothermia: 26.0±3.1%, hyperthermia: 54.6±7.8%, $p<0.001$) and CA1 ($p=0.004$). There was significant less Iba-1 expression in CA1 in hypothermia compared to normothermia and hyperthermia groups ($p=0.001$). No difference existed for GFAP expression in cortex or CA1. **Conclusions:** The enhanced recovery provided by hypothermia and the detrimental effect of hyperthermia were supported by quantitative ischemic neuronal injury in rats after CA. While neuronal preservation was noted more with hypothermia, the brain's immune response also showed decreased microglial activity. Hypothermia may decrease the microglia activation in response of central nervous system to CA than hyperthermia or no treatment in CA1 in this injury model. No difference was noted in the astrocytic activity in the different groups. Supported by NIH R01HL071568 and R21NS054146

PNPH, A NEUROPROTECTANT HBOC: STUDIES OF *IN VIVO* AND *IN VITRO* TRAUMATIC BRAIN INJURY

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The combination of traumatic brain injury and hemorrhagic shock (TBI+HS) is devastating and is an important challenge in both civilian and military resuscitation. Although Hemoglobin-based Oxygen Carriers (HBOCs) are logical candidates for resuscitation in this setting, vasoactivity and neurotoxicity are obvious limiting concerns for clinical translation. Polynitroxylated pegylated hemoglobin (PNPH) is a novel modified bovine Hb that contains 14-15 antioxidant nitroxide moieties along with pegylation that confer putative beneficial properties. We explored the effects of PNPH in three models relevant to TBI resuscitation, namely 1) experimental TBI in mice followed by 90 min of mild volume-controlled HS and resuscitation (Dennis et al, J Neurotrauma 2008), 2) TBI in mice followed by 35 min of severe pressure controlled HS and resuscitation, and 3) *in vitro* studies using rat cortical neurons alone or exposed to an excitotoxic insult (glutamate/glycine). In both *in vivo* models, PNPH outperformed either Lactated Ringers (LR) or Hextend (HEX) as resuscitation fluids with regard to resuscitation volume required and/or blood pressure achieved. PNPH also exhibited favorable effects on both recovery of brain tissue PO_2 ($PbtO_2$) vs LR, and neuronal death in vulnerable hippocampus vs LR or HEX—as assessed using fluorojade staining. Finally, in neuronal culture, unlike native bovine Hb, PNPH was not toxic across a wide range of concentrations and surprisingly attenuated excitotoxic neuronal death—as assessed using either LDH release or MTT. We conclude that PNPH is a unique small volume resuscitation solution in experimental TBI+HS that exhibits neuroprotective properties both *in vivo* and *in vitro*. Given the possibility of extravasation of HBOCs into brain tissue in TBI or polytrauma resuscitation, PNPH or related nitroxylated Hbs may represent very attractive novel small volume oxygen therapeutic agents.

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RECOMBINANT HEMOGLOBINS AS RESUSCITATION FLUIDS IN A MOUSE MODEL OF TRAUMATIC BRAIN INJURY PLUS HEMORRHAGIC SHOCK

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Recombinant hemoglobins (rHbs) hold promise as novel resuscitation agents. Traumatic brain injury (TBI) plus hemorrhagic shock (HS) represent a devastating combination. We have explored the physiological effects of novel recombinant octameric Hbs in our 3-phase mouse model of TBI + HS, modified to have a HS phase of 35 min, a Pre-Hospital Phase of 90 min, and a Hospital Phase of 15 min. After a controlled cortical impact TBI, a brain tissue PO_2 ($PbtO_2$) probe was inserted. HS was induced with blood withdrawal (2.3 ml/100 g) over 15 min. Mean arterial pressure (MAP) was maintained at 25–27 mmHg until the beginning of the Pre-Hospital Phase. Mice were then assigned to Lactated Ringers (LR) or rHb groups (n = 6–9/group). Three octameric rHbs have been studied: rHb with normal O_2 affinity [NA-Hb(αN78C)], rHb with high O_2 affinity [HA-Hb(αL29F/αN78C)], and rHb with low O_2 affinity [LA-Hb(αL29W/αN78C)]. After bolus LR or rHb (20 ml/kg), additional LR or rHb (10 ml/kg/5 min) was given (if MAP < 70 mmHg) through Pre-Hospital Phase. The Hospital Phase commenced with return of shed blood. Pre-Hospital resuscitation fluid volume was > 4-fold higher in LR group ($p < 0.01$). MAP normalized only in the rHb groups ($p < 0.01$, vs LR). Total blood Hb in rHb groups was ~2–3 g/dl higher vs LR ($p < 0.05$). Lactate was normalized in all groups, lowest in HA-Hb group ($p < 0.05$). $PbtO_2$ was numerically lowest in HA-Hb group. Octameric rHbs normalized MAP and lactate with much smaller volume than LR after TBI + HS. Differences in Hb oxygen affinity could not account for their vasoactivity, but may impact $PbtO_2$. Current studies are examining the effect of these novel rHbs on neuronal death and brain edema in TBI resuscitation.

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P361

NON-CLINICAL RESULTS OF THE ECE/NEP INHIBITOR SLV334, A NEW THERAPEUTIC CLASS TO BE INVESTIGATED IN TRAUMATIC BRAIN INJURY (TBI)

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Introduction: SLV334, a metalloprotease inhibitor, is in clinical development for TBI. It inhibits endothelin converting enzyme (ECE), preventing formation of the vasoconstrictor endothelin-1 (ET-1) from its precursor Big endothelin-1 (Big-ET-1). SLV334 also inhibits neutral endopeptidase (NEP), preventing the breakdown of natriuretic peptides, i.e. atrial natriuretic peptide (ANP).
Purpose: To show the effect: on enzyme inhibition; on secondary damage, at the site of impact and also on impaired motor and cognitive function seen in animal models after TBI.
Methods: SLV334's enzyme inhibition was studied *in vitro* and its functional antagonistic activity in a Big-ET-1 induced constrictory response in a rabbit saphenous artery assay and in Big-ET-1 induced hypertension in anesthetized rats. Subsequently, SLV334 was tested in experimental models of TBI in rats. Cortical damage and hippocampal CA3 damage were examined in a controlled cortical impact/weight drop (CCI) model, 3 days post-injury. Moreover, the effect on impaired vestibular motor (beam walking on day 1, 3, 7 post-injury) and cognitive function (Morris water maze on day 14–17 post-injury) was studied in a fluid percussion injury (FPI) model.
Results: SLV334 inhibited the enzymatic activity of hECE-1 (from Sf9 cells; IC_{50} 374 nM) and NEP (from guinea-pig cortex; IC_{50} 4 nM) *in vitro* and antagonized the Big-ET-1 induced constriction in the artery assay (IC50 ~ 1 nM) and hypertension (ID_{50} ~ 17 mg/kg iv bolus). Injection of SLV334 (10 μg ~0.02 μmol icv and 30, 100 mg/kg iv bolus) 15' post-CCI-injury elicited 10–20% significant reduction of neuronal loss in the CA3 subfield of the hippocampus. SLV334 (100 mg/kg iv bolus) tested 4 h post-injury also showed significant protection. SLV334 (100, 300 mg/kg iv bolus) 4 h post-CCI-injury showed significant reduction in the amount of cortical damage at the site of impact. The same dose 4 h post-FPI-injury significantly reduced the trauma-induced deficits in motor function and cognitive function. SLV334 (300 mg/kg iv bolus) also tested after extended periods post-injury (8, 24, 48 h) was effective up to 24 h post-injury.
Conclusion: SLV334's enzyme inhibition was shown in pharmacology studies; in TBI models, SLV334 not only prevented secondary damage in the hippocampus, but was also neuroprotective at the site of impact, and improved motor and cognitive function after TBI, even after administration up to 24 hours after injury. The overall results in two different non-clinical TBI models suggest that SLV334 might have therapeutic potential in the treatment of TBI in humans.

P362 — STUDENT COMPETITION FINALIST

POLOXAMER P188 ATTENUATES CELL MEMBRANE PERMEABILITY, AND IMPROVES HISTOPATHOLOGICAL AND FUNCTIONAL OUTCOME FOLLOWING TRAUMATIC BRAIN INJURY IN MICE

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Background: Loss of plasma membrane integrity after traumatic brain injury is a marker of cell death. Poloxamer P188 (P188) is a non-ionic copolymer that promotes membrane resealing in injured cells. We tested the hypothesis that P188 reseals damaged cell membranes and promotes histopathological and functional recovery following controlled cortical impact (CCI) in mice.
Methods: Adult CD1 or C57/BL6 mice were administered the green fluorescent cell membrane impermeant dye YOYO-1 intravenously (IV) immediately before CCI. At 1 hour, P188 (5 mM, 20 ml/kg) or PBS was administered IV. Propidium iodide (PI) was administered IV at various times after injury and mice were killed 10 min later. Resealed cells were identified as YOYO-1 + /PI-. Brain edema was assessed at 24 h by the wet-dry weight method, blood brain barrier (BBB) leakage (1–24 h) was quantitated using Evans Blue, and lesion size was determined by image analysis at 2 weeks after CCI. Motor and cognitive function was determined by wire grip and Morris water maze tests, respectively.
Results: P188 induced plasma membrane resealing in over 50% of initially permeabilized cells in injured cortex and hippocampus. Spontaneous membrane resealing was observed after 6 h in the absence of P188. P188 also reduced brain edema by 45% ($p < 0.005$), BBB leakage by 94% ($p < 0.005$), brain tissue loss by 29% ($p < 0.05$), motor deficits ($p < 0.05$ group effect), and improved cognitive function ($p < 0.05$ vs. vehicle) after CCI. In PI-pulse labeling experiments designed to follow the fate of injured cells over time, P188 did not rescue injured cells from eventual death after CCI.
Conclusions: Postinjury administration of P188 reseals permeable cell membranes and improves clinically relevant outcome measures after CCI in mice. These beneficial effects are not associated with long term survival of resealed cells in brain, implicating mechanisms other than rescue of injured cells per se.

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MITOCHONDRIAL PROTECTION AFTER TRAUMATIC BRAIN INJURY BY SCAVENGING LIPID PEROXYL RADICALS

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Lipid peroxidation (LP)-mediated oxidative damage is initiated after traumatic brain injury (TBI) by the action of reactive oxygen and reactive nitrogen species (e.g. peroxynitrite), and is catalyzed by an iron-dependent mechanism. However, lipid peroxyl radicals ($LOO^{\cdot}$) are the central propagators of LP reactions across cellular and mitochondrial membranes leading to irreversible loss of mitochondrial respiration, oxidative phosphorylation and ion transport which is implicated in neuronal cell death. The aim of this study was to pharmacologically validate the role of lipid peroxyl radicals in post-traumatic LP in cortical tissue and mitochondria and define its contribution to the loss of mitochondrial bioenergetics after controlled cortical impact (CCI-TBI) in male CF-1 mice using the potent and selective lipid peroxyl radical scavenger U-83836E (Hall et al. J. Pharmacol. Exp. Ther. 258:688–694, 1991). Two sets of mice were randomized into sham, saline-treated and U-83836E treated (3.0 mg/kg) groups. The sham group received only craniotomy with no further treatment, whereas both saline- and U-83836E-treated groups received craniotomy and were subjected to severe (1.0 mm impact depth) CCI-TBI followed by I.V (tail vein) injection of the assigned treatment at 15 minutes post-injury. The first set of animals was sacrificed at 3h post-injury to analyze oxidative damage markers in cortical tissue homogenates. The injury caused a dramatic increase in both 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT) which are specific markers of LP and protein nitration respectively. The drug significantly reduced levels of 4-HNE and 3-NT in cortical tissues. In the second set of animals the injury caused a significant increase in 4-HNE and 3-NT and a marked reduction in mitochondrial state III respiration rate (ATP synthesis capacity) in cortical mitochondria harvested at 12 hrs post-injury. U-83836E treatment was able to significantly attenuate levels of 4-HNE and 3-NT and salvage mitochondrial respiratory function. These findings help to validate lipid peroxyl radicals as an important mediator of post-traumatic oxidative damage and mitochondrial dysfunction. They also suggest that LP contributes to peroxynitrite-mediated protein nitration. Future work is aimed at exploring whether selective scavenging of lipid peroxyl radicals will mitigate neurodegeneration after TBI.

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P227

EFFECT OF SELECTIVE BRAIN COOLING ON ACUTE NEUROPATHOGICAL CHANGES FOLLOWING PENETRATING BALLISTIC-LIKE BRAIN INJURY IN RATS

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Hypothermia is clinically neuroprotective against brain injury, but systemic side effects, such as hypotension, can be caused by whole body cooling. We previously reported that selective brain cooling (SBC) significantly attenuates the acute elevation of intracranial pressure (ICP) following penetrating ballistic-like brain injury (PBBI) in rats. To determine the neuroprotective effect of SBC on acute neuropathological changes following PBBI, we measured lesion size, hemorrhage, brain edema, blood-brain barrier permeability, and neurological functional recovery at 24 h post-PBBI. SBC (3°C below the baseline brain temperature) was induced in anesthetized rats within 30 min after 10% right frontal PBBI by extraluminal cooling of bilateral common carotid arteries (CCA) using cooling cuffs, and maintained for 2 h before spontaneous re-warming was allowed. The body temperature was regulated at 37°C using a heating blanket. The control rats received either PBBI or probe penetration only without SBC. The results showed that SBC significantly reduced PBBI-induced increases in lesion size, hemorrhage, brain edema, and BBB permeability to albumin (by 40.3%, 43.1%, 44.6% and 44.2%, respectively) at 24 h post-PBBI. Results of neurological score assessments also showed that SBC promoted significant functional recovery 24 h post-PBBI compared to untreated PBBI injured rats ($p < .05$). These results demonstrated a neuroprotective effect of SBC on PBBI. Current studies are underway evaluating longer recovery times and cooling periods as a possible therapeutic strategy for severe TBI.

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RESUSCITATION OF TRAUMATIC BRAIN INJURY AND HEMORRHAGIC SHOCK WITH CRYSTALLOID AND COLLOID THERAPIES: EFFECTS ON ACUTE RESUSCITATION PARAMETERS, SURVIVAL, AND NEURONAL DEATH

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Outcome after traumatic brain injury (TBI) is worsened by hemorrhagic shock (HS), and the choice of treatment with crystalloid or colloid therapy is the subject of intense debate. Commonly used fluids include crystalloid lactated Ringers (LR), and colloids human serum albumin (HSA) and Hextend (HEX). Polynitroxyl albumin (PNA, Synzyme) is a novel colloid composed of an albumin molecule with 55 nitroxyl moieties. PNA, alone or administered with Tempol (PNA+), had favourable effects in HS, stroke, and cerebral hemorrhage models (Kentner, 2002). We hypothesized that resuscitation with the colloids HEX, HSA, PNA, or PNA+ is equal to or better than resuscitation with the crystalloid LR. Isoflurane anesthetized C57BL6 mice (n = 40) underwent controlled cortical impact (CCI) and 90 min of HS (2.0cc/100g, MAP ~35mmHg). Mice were randomized to resuscitation with LR, HEX, HSA, PNA or PNA+, followed by 30 min of test fluid administration targeting MAP >50 mmHg (prehospital phase). Shed blood was returned and MAP > 60 mmHg targeted (hospital phase). MAP, fluid volume required, 7d survival, and hippocampal CA1 and CA3 neuron counts were assessed. MAP at the end of HS did not differ between groups. HEX, HSA, PNA, PNA+ achieved significantly higher MAP in the prehospital phase vs LR (58.35 ± 1.27 mmHg, 59.65 ± 1.23 mmHg, 60.69 ± 1.39 mmHg, 69.15 ± 1.69 mmHg, vs. 50.27 ± 1.84 mmHg, respectively, ANOVA $P < 0.01$). Prehospital and total fluid volumes required were significantly less for HEX, HSA, PNA, and PNA+ vs LR (0.23 ± 0.02ml, 0.14 ± 0.01ml, 0.17 ± 0.02ml, 0.26 ± 0.04ml vs. 0.81 ± 0.07ml, respectively, ANOVA $P < 0.01$). No group required fluid administration during the hospital phase. There was no significant difference in survival at 7 days (5/8 LR, 6/8 HEX, 6/8 HSA, 5/8 PNA, 7/8 PNA+). CA1 neuronal loss did not differ significantly between groups (LR 29.24 ± 5.56%, HEX 35.68 ± 6.51%, HSA 44.41 ± 3.75% PNA 34.34 ± 5.72%, PNA+ 21.85 ± 8.21%). CA3 neuronal loss also did not differ between groups. Our data suggest that colloids HEX, HSA, PNA, and PNA+ confer acute benefit without deleterious effects on survival and hippocampal neuronal death vs. crystalloid LR after combined TBI + HS. Further studies assessing the effects of these therapies on ICP and cognitive outcome are currently underway.

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P229

EFFECTS OF MINOCYCLINE ON MICROGLIAL ACTIVATION AND DIFFUSE AXONAL INJURY FOLLOWING TRAUMATIC BRAIN INJURY IN MICE

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Introduction: Minocycline has shown to exert anti-inflammatory and neuroprotective effects in several animal models of neurodegenerative diseases and acute brain injuries. However, the effect of minocycline on the consequences of traumatic brain injury (TBI) is still not fully investigated. Since neuroinflammation and diffuse axonal injury (DAI) are two consequences of TBI, we investigated the effects of minocycline on the TBI-induced microglial activation, and axonal accumulation of β-amyloid precursor protein (β-APP) as markers of neuroinflammation and DAI (1), respectively.
Methods: The weight drop model was used to induce TBI in mice (2). Microglial activation and DAI were evaluated by immunohistochemistry using CD11b and β-APP markers, respectively. Minocycline was administered either 1) three times 5 min (90 mg/kg, i.p.), 3 and 9h (45 mg/kg, i.p.) following TBI (short-term protocol), or 2) six times 5 min (90 mg/kg, i.p.), 3, 9, 24, 36 and 48h (45 mg/kg, i.p.) following TBI (long-term protocol). The levels of CD11b and β-APP were evaluated at 24 (short-term protocol) or 72h post-TBI (long-term protocol).
Results: The kinetic studies of post-TBI microglial activation and DAI showed an increase of CD-11b immunolabelling as well as an acute axonal accumulation of β-APP from 6h up to 72h post-TBI. While minocycline treatment decreased the elevation of post-TBI CD11b marker in the short-term protocol ($P < 0.05$), it was devoid of effect in the long-term protocol. Besides, minocycline had no significant effect on the TBI-induced axonal accumulation of β-APP in none of the protocols used in this study.
Conclusion: Treatment with minocycline was able to reduce the TBI-induced microglial activation in the short-term protocol. However, it was not able to reduce the traumatic axonal injury suggesting that under our experimental conditions the microglial activation does not affect DAI following TBI.
1) Stone et al. Brain Res. 2000;871(2):288–302.
2) Hellal et al. J Neurotrauma. 2003;20:841–51.

P230

PNPH, A NEUROPROTECTANT HBOC: STUDIES OF *IN VIVO* AND *IN VITRO* TRAUMATIC BRAIN INJURY

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The combination of traumatic brain injury and hemorrhagic shock (TBI + HS) is devastating and is an important challenge in both civilian and military resuscitation. Although hemoglobin blood oxygen carriers (HBOCs) are logical candidates for resuscitation in this setting, vasoactivity and neurotoxicity are obvious limiting concerns for clinical translation. Polynitroxylated pegylated hemoglobin (PNPH) is a novel bovine Hb that contains 14–15 antioxidant nitroxide moieties along with pegylation that confer putative beneficial properties. We previously reported beneficial effects of PNPH in an established model of volume controlled HS after CCI in mice. We now explore the effects of PNPH in two models relevant to TBI resuscitation, namely 1) TBI in mice followed by 35 min of severe pressure controlled HS and resuscitation, and 2) *in vitro* studies using rat cortical neurons alone or exposed to an excitotoxic insult (glutamate/glycine). In the *in vivo* model, PNPH outperformed Lactated Ringers (LR) as a resuscitation fluid with regard to resuscitation volume required and blood pressure achieved ($p < 0.05$). PNPH also exhibited favorable effects on both recovery of hippocampal brain tissue PO_2 tension ($PbtO_2$) vs LR ($p < 0.05$), and neuronal death in vulnerable regions of the hippocampus vs LR ($p < 0.05$). Finally, *in vitro* studies showed that unlike native bovine Hb, PNPH was not toxic across a wide range of concentrations ($p < 0.05$ vs bovine Hb) and surprisingly attenuated excitotoxic neuronal death—as assessed using either LDH release or MTT. Remarkably, PNPH (but not control bovine Hb) also attenuated glutamate/glycine-mediated neuronal death in culture ($p < 0.05$ vs glutamate/glycine). We conclude that PNPH is a unique small volume resuscitation solution in experimental TBI + HS that exhibits neuroprotective properties both *in vivo* and *in vitro*. Given the possibility of extravasation of HBOCs into brain tissue in TBI or polytrauma resuscitation, PNPH or related nitroxylated Hbs may represent very attractive novel small volume oxygen therapeutic agents.

Support: US Army PR054755W81XWH06-01-0247 and NS30318.

Print Close

A235
October 17, 2009
2:00 PM - 4:00 PM
Room Area I

Novel Recombinant Hemoglobins (rHb) for Traumatic Brain Injury Resuscitation in Mice

** Xianren Wu, M.D., Nancy Ho, David Shellington, M.D., Chien Ho, Ph.D., Patric Kochanek, M.D.
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Introduction: Genetically engineered hemoglobin (Hb) products hold promise as novel resuscitation agents for trauma patients. Traumatic brain injury combined with hemorrhagic shock (TBI+HS) represents a devastating combination. Our recent studies demonstrated that Hb based products have potential for neuroprotection in resuscitation of TBI+HS (1). We now explored the physiological effects of novel recombinant octameric Hbs in our mouse model of TBI+HS.

Methods: A 3-phase mouse TBI+HS model (2) was modified to have a HS phase of 35 min, a Pre-Hospital Phase of 90 min, and a Hospital Phase of 15 min. After a standardized controlled cortical impact (CCI) to the left parietal cortex was delivered, a PO_2 probe was inserted through the left frontal craniotomy to a depth of 2 mm into dorsal hippocampus. HS was induced with blood withdrawal (2.3 ml/100g) over 15 min. Mean arterial pressure (MAP) was then maintained between 25-27 mmHg for additional 20 min. At the beginning of the Pre-Hospital Phase, mice were assigned to LR (n=6) or rHb groups (n=7-9 each group). Three rHbs were studied: rHb with normal O_2 affinity (NA-Hb)(αN78C), rHb with high O_2 affinity (HA-Hb)(αL29F+αN78C), and rHb with low O_2 affinity (LA-Hb) (αL29W+αN78C). After an initial bolus of LR or rHbs (20 ml/kg), a continuous infusion of LR at 20 ml/kg/h was started. Additional LR or rHb solutions (10 ml/kg per 5 min) was given if MAP was <70 mmHg through the Pre-Hospital Phase. The Hospital Phase was started with 100% O_2 and return of all shed blood. Mice were observed to 24 h.

Results: There were 1-2 animal deathes during the Pre-Hospital Phase in each group. The total volume of resuscitation fluid during the Pre-Hospital Phase was 50±0 ml/kg in rHb groups, and 198±30 ml in the LR groups ($p<0.01$). MAP was normalized only in the rHb groups ($p<0.01$, vs LR).[figure1]The initial MAP in the NA-Hb group was higher than other two rHb groups ($p<0.05$). At the end of the Pre-Hospital Phase, total blood Hb levels in rHb groups were about 2-3 g/dl higher than that in the LR group ($p<0.05$). Lactate levels were normalized in all groups with the lowest level in the HA-Hb groups ($p<0.05$). Brain tissue O_2 was numerically lowest in the HA-Hb group ($p>0.05$).[figure2] **Conclusion:** Octameric rHbs normalized systemic blood pressure and systemic metabolism with much smaller volume than LR in resuscitation of TBI+HS in mice. Differences in oxygen affinity of Hbs could not account for their vasoactivity, but may impact brain tissue PO_2.

References:

1. Shellington D, et al. Crit Care Med 36(Suppl):A5, 2008

2. Dennis AM, et al. J Neurotrauma. 2008 [Epub].

From Proceedings of the 2009 Annual Meeting of the American Society Anesthesiologists.

Figure 1

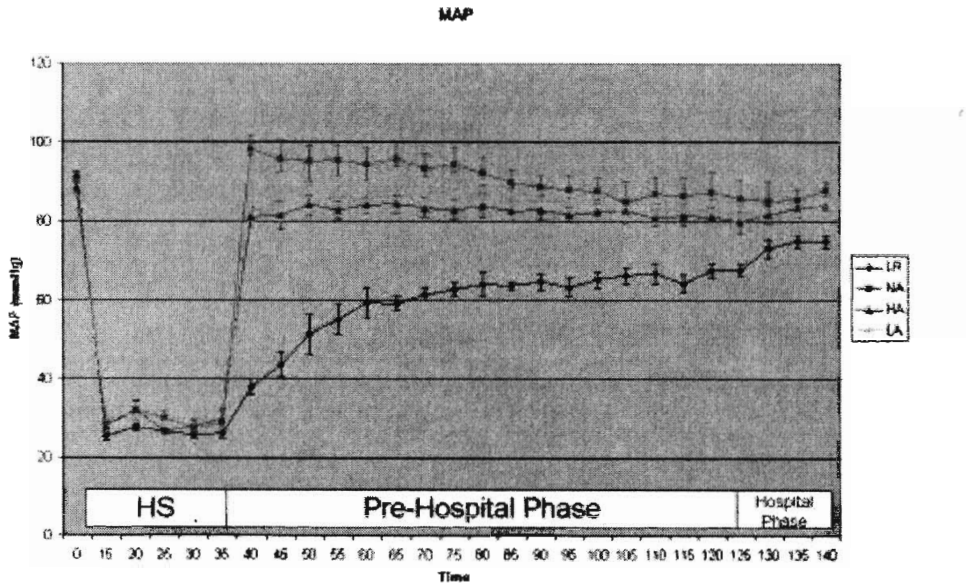
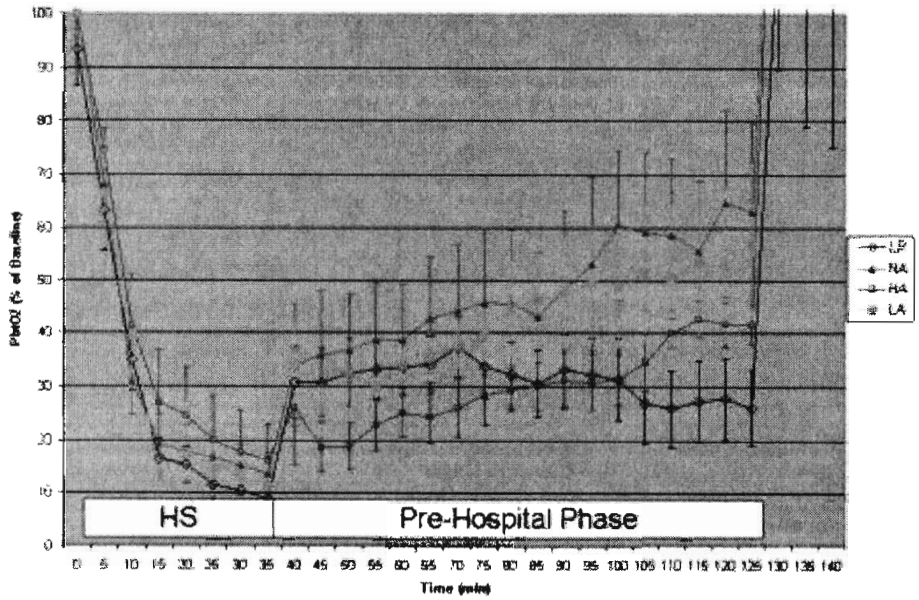

MAP
HS
Pre-Hospital Phase
Hospital Phase
LR
NA
HA
LA
Time

Figure 2


HS
Pre-Hospital Phase
LR
NA
HA
LA

Print Close

A241
October 17, 2009
2:00 PM - 4:00 PM
Room Area I

Polynitroxylated Pegylated Hb (PNPH) in Resuscitation of Traumatic Brain Injury + Hemorrhagic Shock

** Xianren Wu, M.D., Lina Du, M.D., Li Ma, Ph.D., David Shellington, M.D., Patrick Kochanek, M.D.
Anesthesiology, University of Pittsburgh, Pittsburgh, Pennsylvania

Introduction: The combination of traumatic brain injury (TBI) and hemorrhagic shock (HS) poses a special challenge to resuscitation medicine. Previously we found promising neuroprotective effects of PNPH (bovine) in resuscitation of TBI plus HS in mice. However, hemoglobin (Hb) that leaks into the brain parenchyma after TBI is expectedly neurotoxic. The current study explored possible mechanisms that underlie the benefits of PNPH both *in vivo* and *in vitro* .

Methods: *In vivo Study :* We used an established model of combined TBI plus HS in mice that includes two phases, i.e., a prehospital phase followed by a hospital phase. Brain tissue PO_2 ($PbtO_2$) was monitored ipsilateral to controlled cortical impact TBI. At the beginning of prehospital phase, mice were randomized to lactated Ringers (LR) or PNPH (20ml/kg) groups (n=9 each) to 90 min. Hospital phase was then started with 100% O_2 and return of shed blood. Mice were observed to 24 h and then sacrificed for neuropathology. *In vitro Study:* Primary cortical neuron-enriched cultures were prepared from 16 to 17-day-old Sprague-Dawley rat embryos. Cell vitality [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test] and neurocytotoxicity [lactate dehydrogenase (LDH) levels] were determined 24 h after incubation of cells with bovine Hb or PNPH; Possible neuroprotective effects were evaluated 24 h after incubation of cells with a mixture of glycine/glutamate and individual test Hb agent at concentrations ranging from 0.63 to 12.5 mM.

Results: *In vivo Study :* During prehospital phase, PNPH treatment normalized MAP with ¼ of the volume vs. LR (total 206±20 vs. 51±4 ml/kg, $p<0.01$), while hypotension in the LR group persisted ($p<0.01$). $PbtO_2$ decreased to <20% of baseline in both groups during HS, but improved after treatment and finally reached 64±36% of baseline in the PNPH group. In contrast, initial recovery of $PbtO_2$ deteriorated steadily in the LR group (28±16% at the end of prehospital phase; vs. PNPH, $p<0.05$).[figure1]Neuropathology showed less CA1 neuronal injury in the PNPH group (vs. LR, $p<0.05$).[figure2] *In vitro Study:* At tested concentrations, bovine Hb produced marked neurotoxicity. Remarkably, no neurotoxicity was detected with PNPH. Incubated with glycine/glutamate, bovine Hb did not provid neuroprotection. Surprisingly, PNPH reduced injury by ~50% as compared to bovine Hb.

Conclusion: Our data support multiple potential beneficial effects of PNPH in TBI +HS resuscitation. First, PNPH improved O_2 delivery, reduced volume requirement, and yielded superior hemodynamics vs. LR. Second, PNPH is devoid of the typical neurotoxic effects of Hb and exhibits direct neuroprotective properties vs. excitotoxicity.

From Proceedings of the 2009 Annual Meeting of the American Society Anesthesiologists.

Figure 1

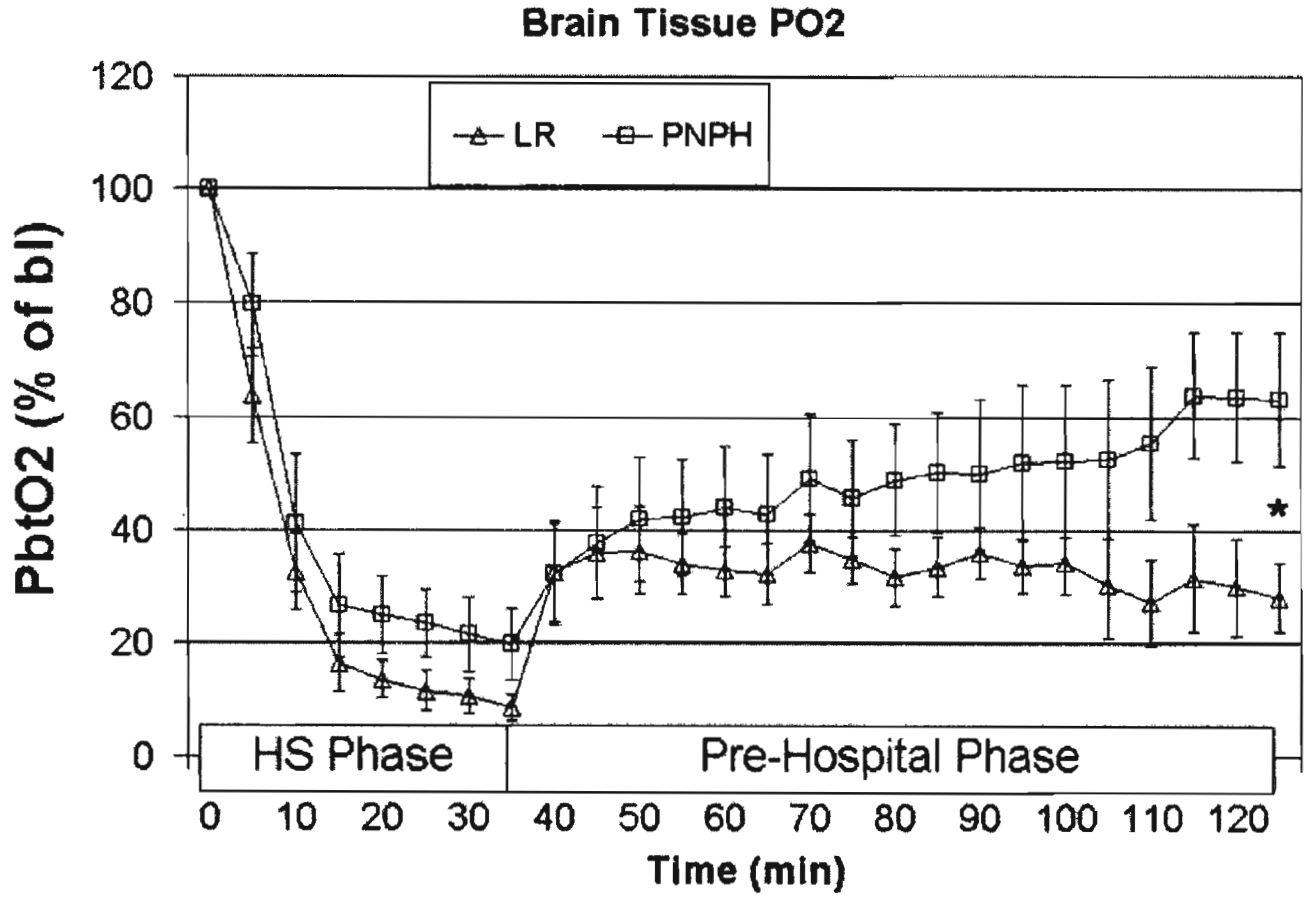
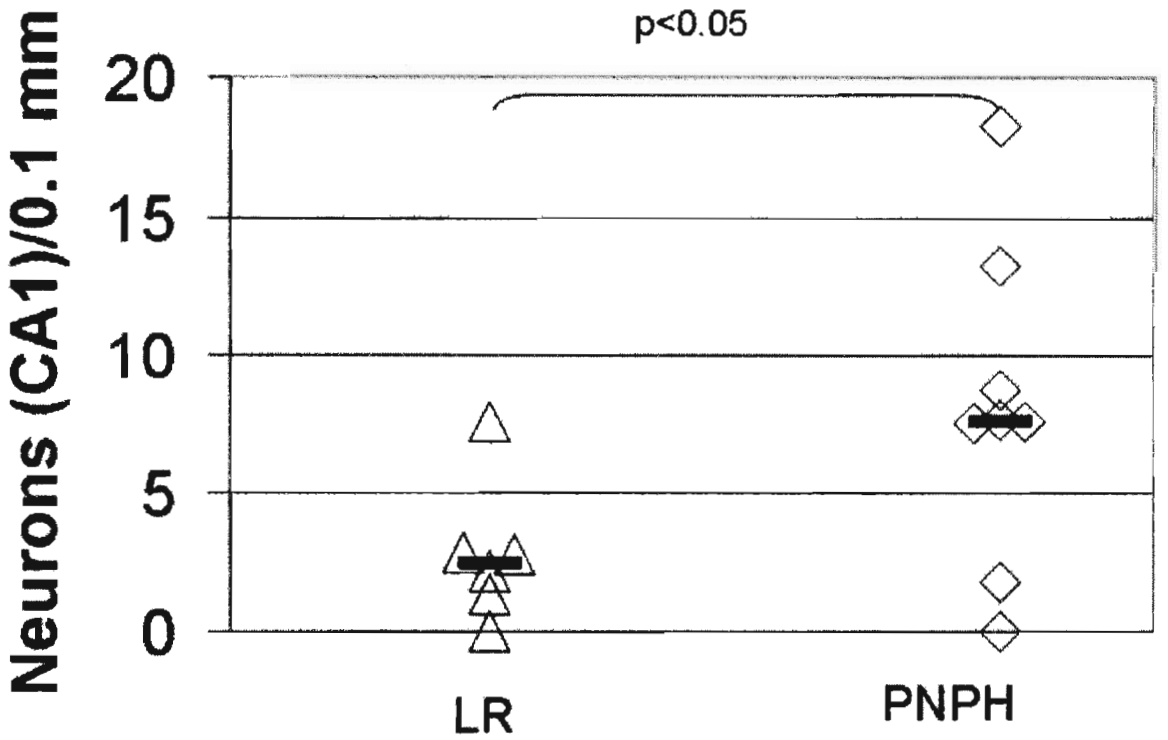

Brain Tissue PO2
LR
PNPH
PbtO2 (% of bl)
120
100
80
60
40
20
0
HS Phase
Pre-Hospital Phase
0 10 20 30 40 50 60 70 80 90 100 110 120
Time (min)
*

Figure 2


p<0.05
Neurons (CA1)/0.1 mm
20
15
10
5
0
LR
PNPH

Reference 30. Proceedings from the 2009 ATACCC Meeting

Novel approaches in resuscitation and therapy of blast TBI and polytrauma

Patrick M. Kochanek, Larry Jenkins, Robert Garman, Robert SB Clark, Lina Du, C. Edward Dixon, Hulya Bayir, Edwin K. Jackson, Valerian Kagan, Li Ma, PhD, Carleton Hsia, Steve Parks, David Ritzel, and Richard Bauman.

Blast-induced traumatic brain injury (TBI) with or without polytrauma has taken on great importance in combat casualty care. We have pursued an enhanced understanding of these processes through studies in both *in vivo* rodent models including the development of a model of air blast injury in rats with thoracic and abdominal protection and the use of a novel mouse model of combined TBI plus hemorrhagic shock (TBI+HS), and an *in vitro* model of neuronal stretch. In studies of blast-induced TBI in rats, we examined an injury level that produced 75% survival and serially assessed brain sections over 2 wks after injury using H&E (conventional neuropathology), Iba-1 (microglial response), GFAP (astrocyte response), and amino curpric silver (for neuronal and nerve process damage) staining. Silver staining was the most sensitive assessment tool and revealed extensive damage to axons and/or nerve terminals in cerebellum, brain stem, and hippocampus. In TBI+HS, neuronal death was exacerbated vs TBI alone. Use of the novel agent polynitroxylated pegylated hemoglobin (PNPH) as a small volume resuscitation solution improved brain tissue oxygen levels in the injured hippocampus and attenuated neuronal death vs conventional fluids such as lactated Ringers or Hextend. Surprisingly, PNPH was also neuroprotective in neuronal stretch in culture—a system where control Hb was neurotoxic. Benefit of PNPH likely is conferred by the covalent linkage of 15 nitroxide moieties. We are also exploring the potential secondary injury cascades with tools such as gene array and oxidative lipidomics and testing other resuscitation strategies and therapies in these models including the impact of hyperventilation and hyper-oxygenation, putative neuroprotective agents such as progesterone, poloxamer-188, deferoxamine, FK-506, and minocycline, along with nutraceuticals such as caffeine, sulforaphane, and resveratrol, among other therapies. In conclusion, our data suggest that rodents can be used to appropriately model blast TBI and polytrauma. Amino cupric silver staining may represent a key tool to examine therapeutic approaches to blast TBI. Finally, novel covalently-modified Hbs, such as PNPH, may represent a paradigm shift in TBI resuscitation. Support: USAMRAA PR054755 W81XWH-06-1-0247 and DARPA.